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THE FAILURE TO DETECT ANTIDIURETIC SUBSTANCE IN THE BLOOD AND URINE OF DEHYDRATED RATS

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Inexplicable variations in the concentration of antidiuretic hormone in serial specimens of urine collected over periods of six hours from normal and toxaemic patients during pregnancy and the puerperium led us to review the whole problem from several angles. In another paper (1951) we report the results of studies on the effect of growth of *Bact. coli* (human strains) in urine. Unexpectedly we found that frequently an antidiuretic response could be obtained from concentrates of such urine.

At the same time we re-investigated the method of assay and also tried to repeat the experiments of Gilman and Goodman (1937), the results of which appeared to have been confirmed by Teel and Reid (1939). We had previously assumed that Burn's method (1931) was satisfactory because it had been accepted by many workers and we ourselves had apparently successfully used it to assay antidiuretic activity in urine from toxaemic pregnant women.

EXPERIMENTAL.

Experiments to test the reliability of the method of assay.

We followed the instructions of Burn (1931) as far as the method was concerned, but modified the assessment for the results as described by us in previous papers (1940, 1946) by measuring area differences subtended by graphs obtained when the total volumes of urine excreted by control rats and by treated rats over a period of five hours, are plotted against time. The units of ordinate and abscissa were 60 minutes per inch and 10 ml. per inch respectively. An area difference of 5·6 square inches had previously been found to be the minimum value representing a true antidiuretic effect in experiments under the conditions specified in our first paper (1940). Although the period for observation of the volume of urinary secretion could be shortened to three hours, we wished to keep these experiments comparable with the earlier ones. Standard dilutions of various commercial preparations of pituitary extract were made in isotonic saline. Sixteen male rats, all of which were given water to the extent of 5 p.c. of their body weight by a stomach tube, were divided into three experimental and one control group. In the control group each of the rats received an intravenous injection of 0·5 ml. of

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isotonic saline, whilst those in the experimental groups were similarly injected with 0·5 ml. of pituitary solution.

Of the commercial extracts investigated, that supplied by Burroughs, Wellcome and Co. ("Infundin," containing 10 international units per ml. of posterior lobe extract) was the only preparation which we found to contain relatively constant amounts of antidiuretic hormone. In addition graded doses of this extract gave the corresponding variations in inhibition of water diuresis. The finding of Heller and Urban (1935) that a certain concentration of the extract will produce a maximum effect beyond which further increase in antidiuretic activity will only occur if massive doses of pituitary extract are given, was confirmed by these experiments.

TABLE 1.

Triplicate estimations of antidiuretic hormone in posterior pituitary extracts (Burroughs Wellcome), showing the reliability of the method of assay.

Batch No.	Assay by method of area differences		
	Square inches	Square inches	Square inches
1	11·9	10·9	12·1
2	12·1	14·1	15·5
3	9·4	9·6	10·6
4	12·7	14·2	12·8
5	16·3	16·3	15·3
6	10·5	12·1	12·1
7	12·6	14·3	15·4
8	15·1	16·1	17·4

In eight experiments in which triplicate assays were performed on different samples of extract, the antidiuretic activity was remarkably constant (Table 1). The individual experiments showed variations which might be ascribed to differences in antidiuretic activity in various batches of "Infundin."

Repetition of Gilman and Goodman's dehydration experiments.

Having proved that the method of assay is, in fact, reliable, we now attempted to show the presence of antidiuretic principle in the urine from dehydrated rats in order to test the claim of Gilman and Goodman (1937) that if the necessity for water retention was produced in rats either by withholding water for several days or by the ingestion of suitable amounts of 5 p.c. sodium chloride, the urine voided by such animals contained an antidiuretic substance. In repeating the work of Gilman and Goodman, two groups, each consisting of four rats, were placed in metabolism cages supported in large funnels. One set, which had free access to water, acted as the control group for the second set which was deprived of water for 72 hours. The urine collected from each set was assayed for antidiuretic substance and the results are recorded in Table 2.

In thirty assays included in fifteen such experiments significant amounts of antidiuretic substance were obtained from the urine in only six instances. In contrast to the observations of Gilman and Goodman, antidiuretic substance was found only once in the urine voided by the dehydrated rats, whereas it occurred in that from five sets of rats in water equilibrium.

Further, the amount of antidiuretic substance found in the urine from this set of dehydrated rats was much less than that in the urine obtained from the control group. These results were unexpected in view of the fact that the urine excreted by the dehydrated rats was always about one sixth of that passed by the controls.

TABLE 2.

A comparison of the antidiuretic activity in the urine of hydrated and dehydrated rats.

Experiment No.	Antidiuretic activity in the urine of rats Measured in square inches	
	Dehydrated rats	Hydrated rats (control)
1	4·2	3·3
2	6·6	10·0
3	2·2	11·9
4	2·3	6·4
5	0	0
6	3·9	0
7	2·8	3·0
8	0	5·6
9	0	2·0
10	0	0
11	2·2	4
12	0	0
13	0	0
14	0*	0
15	2·5*	5·9

* Dehydration was obtained by ingestion of 5 ml. of 5 p.c. NaCl. per 100 gm. body weight.

For interest Gilman and Goodman's method of computing antidiuretic activity was applied to these experiments and the results in no way differed from the findings using our method.

Our failure to obtain antidiuretic effects in the urine of dehydrated rats may, however, have been due to contamination from rat faeces as suggested by Gilman and Goodman since such contamination was not avoided in the method of collection of urine used in these experiments.

The effect of rat faecal contamination on antidiuretic activity.

A series of experiments was designed to determine the effect which deliberate contamination by rat faeces would have on antidiuretic activity. The two factors which might account for the decrease in activity caused by rat faeces, as suggested by Gilman and Goodman are, firstly, bacterial action, and secondly, adsorption of the antidiuretic principle on to the faecal matter. The effect of each of these factors was tested by incubating three solutions containing the same amount of pituitary extract for 48 hours (a) alone, (b) with faeces, (c) with a second portion of the same faeces sterilized by autoclaving. In these experiments the effects of solid faeces, macerated faeces and saline extracts of macerated faeces were all investigated.

Results.

The results of these experiments are recorded in Table 3 which shows that, in general, there is a decrease in antidiuretic activity when pituitary extract is incubated with non-autoclaved rat faeces, or a saline extract. In at least 10 of the 24 assays a significant decrease in activity was obtained, and in every case, with the exception of four assays when the faeces were in pellet form, some diminution was noted. On the other hand when the faeces were first sterilized by autoclaving no significant reduction took place except in one instance (exp. 15).

TABLE 3.

The effect of faecal contamination on pituitary antidiuretic substance.

Experiment No.	Antidiuretic effect measured in square inches.			
	Pituitary control	Pituitary with rat faeces	Pituitary with autoclaved rat faeces	
1	8·6	3·3	Not assayed	Solid Pellets
2	8·9	7·1	6·3	
3	9·9	15·1	10·5	
4	6·4	11·5	5·5	
5	8·3	13·2	5·7	
6	6·3	12·3	6·7	
7	13·3	7·0	13·1	Macerated faeces
8	16·8	6·3	14·4	
9	12·1	Not assayed	8·3	
10	10·1	4·4	9·8	
12	12·0	8·6	11·5	
13	12·3	6·2	12·3	
14	13·8	8·0	10·6	
15	11·1	1·2	4·6	
16	13·8	5·3	9·1	
17	12·7	11·3	9·6	
18	9·7	6·1	9·5	
19	10·3	Not assayed Duplicates	11·9 12·9	
20	11·7	6·8	12·2	Saline Extracts of Macerated Faeces
21	14·0	9·4	13·3	
22	11·1	7·9	13·0	
23	8·3	5·6	11·4	
24	6·2	2·8	10·2	
25	9·9	2·2	12·2	

From these observations it is apparent that bacterial action and not adsorption phenomena, is responsible for the loss of antidiuretic activity of the pituitary extract. In addition, these results support the claim of Gilman and Goodman that unless rat faecal contamination is avoided, the pituitary antidiuretic principle measured in the assays may be less than that actually excreted in the urine by the dehydrated rats.

Repetition of Gilman and Goodman's dehydration experiments in which bacterial contamination is controlled.

(a) *Method of collecting specimens.*

Originally, the rats from which urine was to be collected were placed in metabolism cages with a fine wire mesh base supported in a large funnel. The urine voided by the rats passed into a flask placed under the stem of the funnel. The metabolism cages had, however, rather coarse latticed sides and during the long period of confinement in these small cages, accumulated faeces had a tendency to escape and collect in the narrow outlet of the funnel and sometimes fall into the collecting vessel. This led to gross contamination in many specimens. To overcome this difficulty fine mesh wire discs about 3 inches in diameter were carefully fitted

into the lower part of the funnel. These discs were effective in retaining not only the faeces but also large amounts of hair and other debris. Despite this precaution, contamination still resulted.

It therefore became necessary to apply some means of inhibiting bacterial growth occasioned by such contamination. Obviously the use of the usual bacteriostatics was undesirable. A method of extreme cooling seemed likely to be the most satisfactory since a series of experiments had proved that such treatment did not diminish the principle being assayed. Temperatures of the order of —60° C. were obtained by using a mixture of ethyl alcohol and small pieces of carbice (solid CO_2) in Dewar flasks. The collecting tube was immersed in this freezing mixture and the urine was instantaneously solidified when it ran into the tube. The actual apparatus used consisted of 6 in. × 1 in. test tubes which just fitted the neck of 1 pint thermos flasks. The tubes were held in position by cork discs. Care had to be taken to ensure that the drops of urine fell vertically into the collecting tube, otherwise the urine solidified on the cooled upper walls of the tubes and formed a "shelf" upon which subsequent specimens accumulated. During hours when it was impossible to remove specimens at intervals, the available space in the tubes was sometimes insufficient to collect the whole of the urine which was secreted.

Another technical difficulty arose in efforts to protect the collected samples from air borne contamination. Small glass funnels were inserted in the test tubes but the urine froze in the narrow stems of these funnels. However, it has been shown that any air borne contamination entering the open mouth of the collecting tube would have little, if any, effect on the solidified urine.

Experimental results.

In twenty-four experiments in which urine was collected at room temperature for periods up to seventy-two hours, all specimens showed bacterial contamination; *Bact. coli* (rat strain) was present in twelve instances. Contamination due to this organism was gross in nine of these twelve samples. As we have already shown that *Bact. coli* from rat faeces is able to destroy the antidiuretic activity in pituitary extracts, the occurrence of these organisms has been used as an index of the efficiency of this method for collecting specimens.

TABLE 4.

Correlation of the Bact. coli *contamination in specimens of urine from dehydrated and hydrated rats collected at —60° C.*

Experiment No.	*Bact. coli* in specimens from dehydrated rats			*Bact. coli* in specimens from hydrated rats	
	(1)	(2)	(3)		
1	+	±	±	+++	Not completely frozen
2	—ve	—ve	—ve	+++	
3	±	—ve	—ve	+++	
4	±	±	—ve	+++	
5	—ve	—ve	—ve		
6	—ve	—ve	—ve		

Key: —ve = No *Bact. coli* present. + = Few *Bact. coli.*
± = Very few *Bact. coli.* +++ = Many *Bact. coli.*

In an additional series of thirty-five experiments in which the whole of the specimen was frozen, only three contained an appreciable number of *Bact. coli.* On the other hand, if the voided specimens were not completely frozen as occurred in some instances in which the column

of urine in the collecting vessel rose above the level of the freezing mixture, gross contamination often resulted. Thus in 23 such experiments half of the specimens contained excessive numbers of *Bact. coli.* The fact that bacterial contamination with *Bact. coli* can be controlled by this method is well illustrated in Table 4. Here the results from quadruplicate experiments are set out. Urine from three sets of rats was obtained under conditions of dehydration, whereas that in the fourth set was obtained from rats which had free access to water. This resulted in small volumes of urine being secreted by the dehydrated rats and much larger quantities by those in water equilibrium. Since rats are largely nocturnal in their habits, maximum secretion of urine occurred during the night and thus complete freezing of the specimen from the hydrated rats could not always be maintained. Table 4 shows that gross contamination occurred only in the four instances in which the upper layer of the specimen remained unfrozen.

Still more striking, as one would expect, are the degrees of contamination with *Bact. coli* observed when urines collected by this method are compared with those in which no attempt was made to prevent bacterial growth (Table 5). Even the contamination found in the partially frozen specimens referred to in Table 4 is considerably less than that which occurs when no bacteriostatic precautions are taken.

TABLE 5.

A comparison of the Bact. coli *contamination in urine from rats collected under different conditions.*

Experiment No.	Specimens collected at −60°C.		Specimens collected at room temperature from dehydrated rats
	Bact. coli in specimens from dehydrated rats	*Bact. coli* in specimens from hydrated rats	
1	+	+++	+++++
2	+	+	+++++
3	++	+++	+++++
4	+	+	+++
5	±	±	+++

Key: ± Very few *Bact. coli.* +++ Many *Bact. coli.*
+ Few *Bact. coli.* +++++ Gross contamination with *Bact. coli.*
++ Moderate number *Bact. coli.*

In experiments in which bacterial metabolism is likely to interfere with the assay of biological material, and in which the usual chemical bacteriostatic reagents cannot be used, the method of instantaneous freezing of specimens by alcohol-carbice mixture greatly reduces bacterial growth. The method has the advantage of being simple and inexpensive, and may prove of value to other workers assaying biological material in which bacterial action may cause unexpected results.

(b) *Results of repetition of Gilman and Goodman experiments.*

Using this method for collection of the specimens the dehydration experiments of Gilman and Goodman were repeated, but no antidiuretic activity was detected in 36 such experiments. In eight instances these included three specimens of urine which were collected over the same 72 hour period from three sets of dehydrated rats, and which were assayed on the same day (Table 6).

TABLE 6.

Measurement of antidiuretic activity in the urine collected from dehydrated rats when faecal contamination and bacterial growth are controlled.

Experiment No.	Antidiuretic effect measured in square inches		
	Urine from dehydrated rats		
	Set I	Set II	Set III
1-3	—ve	—ve	—ve
4-6	—ve	—ve	—ve
7-9	1·1	1·8	3·4
10-11	—ve	2·3	Not assayed
12-14	2·7	2·0	4·4
15-17	—ve	—ve	—ve
18-20	—ve	—ve	0·8
21-23	—ve	—ve	0·8
24	—ve		
25	—ve		
26	3·7		
27	2·6		
28	—ve		
29	—ve		
30	—ve		
31	1·2		
32	—ve		
33	—ve		
34	—ve		
35	1·6		
36	0·3		

Ingestion of NaCl.

Thus, even when faecal contamination and bacterial growth were reduced to a minimum, we were unable to verify the claim of Gilman and Goodman concerning the presence of antidiuretic substance in the urine of dehydrated rats.

These results are interesting since in another paper (1951) we have shown that urine in which *Bact. coli* are allowed to multiply contains a substance with antidiuretic properties. This was true in tests on thirteen out of fourteen human strains and also in two out of seven rat strains of *Bact. coli.* We would have expected that we might have detected antidiuretic activity due to this substance in some of our assays of urine from dehydrated rats which was contaminated by *Bact. coli* from rat faeces. Since no antidiuretic activity was detected in such urine specimens we feel confident that our results prove that pituitary antidiuretic substance is not regularly secreted in the urine of rats dehydrated for periods of 72 hours.

Antidiuretic substance in the blood of dehydrated rats.

Despite the fact that no antidiuretic substance could be recovered from the urine of these dehydrated rats, it could still be present in the blood and account for the greatly decreased urinary output by these animals. To determine whether this was actually the case, blood from both dehydrated and

hydrated rats which were used in five of our previous experiments, was assayed for antidiuretic activity using the method of intraperitoneal injection of the serum suggested by Prendergrass, Hodes and Griffiths (1941). The blood was obtained from the external carotid artery of the rats under light ether anaesthesia. We found it unnecessary to use a cannula in the artery since blood could be obtained by simply cutting it and collecting the blood in sterile centrifuge tubes. After adequate centrifuging, sufficient serum for the intraperitoneal injection of 1 ml. into each of four rats could usually be obtained from two animals. We estimated antidiuretic activity in the serum of the dehydrated rats by comparing the urinary output from rats injected with serum from these animals, with that from those injected with serum from a similar group of hydrated rats. In none of the five experiments could any antidiuretic activity be detected.

SUMMARY.

In these investigations no antidiuretic substance was recovered from the blood or the urine of rats which had been dehydrated by withholding water for 72 hours, or by ingestion of sodium chloride even when faecal contamination and bacterial growth had been reduced to a minimum.

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CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

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Until recently it was considered that cystinuria was primarily an error in metabolism leading to the over-production of cystine or to an inability to metabolise it further. The possibility that cystinuria might, at least in some cases, be due to low renal threshold for this amino acid was mentioned by Cleland (1946), and now Dent (1950) has suggested that it is the result of an altered renal threshold for several amino acids. Generally speaking, the plasma amino acids comprise mainly the essential high molecular weight acids, whereas in the urine the low molecular weight amino acids predominate. This difference in distribution is presumably brought about by the kidneys. Yeh *et al.* (1947) using microbiological methods, and Dent (1950) using chromatography have both shown that in some cystinuric subjects there is in addition an excessive excretion of other amino acids, and particularly of arginine and lysine. The renal threshold theory of cystinuria would also help to explain the effect on cystine excretion of metabolically unrelated amino acids such as leucine as found by Cleland (1947).

This paper reports the effect of various amino acids and proteins on the urinary excretion of cystine, methionine and sulphate sulphur in a patient who excreted about 350–450 mg. cystine a day. Specimens of urine from relatives of the patient were also examined.

Experimental Details.

History.

The fourteen year old boy was admitted to hospital with acute abdominal pain and pyrexia, and was operated on for appendicitis. Histological examination showed that the appendix was normal. Three days later a routine examination of the urine showed the presence of cystine crystals. There had been no history of renal dysfunction, but an intravenous pyelogram taken one week after the operation suggested that although no calculus could be detected, the function of the right kidney was subnormal.

The first specimen of urine used for analysis was collected a week after the operation for appendicitis, and this and subsequent specimens all contained crystals of cystine.

Diet.

During the experiments the patient who was convalescing, consumed a diet which each day usually consisted of one egg, two serves of meat, a pint of milk, cereals and vegetables, but little fruit. The total protein intake was about 1·5 gm. per kilo.

Amino acid and protein supplements.

Amino acid supplements were taken in gelatine capsule with breakfast on the morning of the day indicated (see Table 1). Cystine, methionine or cysteine hydrochloride was given in an amount which corresponded in sulphur content to 2·5 gm. methionine. Each dose of the sulphur-free amino acids was the molecular equivalent of 2·5 gm. methionine. (The tyrosine or asparagine was given as a single dose, the glycine as two single doses, while the leucine was inadvertently taken as half a dose on each of two consecutive days.)

The protein supplements were taken as powders with breakfast on the morning of the day indicated (see Table 1) in amounts which corresponded in protein content to two eggs.

Methods.

The methods of analysis were those used in previous experiments (Cleland, 1945, 1946).

RESULTS.

Results are shown in Tables 1, 2, and 3 and Fig. 1.

TABLE 1.

Influence of protein and various amino acids on urinary sulphur in cystinuria.

Date	Substance fed	Volume ml.	Total N gm.	Sulphur (gm.) as Cystine	Sulphur (gm.) as Methionine	Sulphur (gm.) as Sulphate
July 11		880	9·9	0·11	0·03	0·47
12		820	4·2	0·10	0·01	0·22
13	2·5 gm. methionine	1,030	9·3	0·18	0·07	0·68
14		860	6·9	0·15	0·06	0·33
15		950	10·0	0·12	0·04	0·44
16 and 17 combined	2·5 gm. glycine	1,750	11·7	0·31	0·06	0·45
18		1,160	8·9	0·09	0·05	0·45
19	2 gm. cystine	940	8·8	0·12	0·05	0·48
20		1,360	10·5	0·08	0·06	0·69
21		1,340	12·2	0·12	0·07	0·57
22	1·1 gm. leucine	1,520	12·4	0·11	0·07	0·53
23	1·1 gm. leucine	700	6·5	0·12	0·03	0·32
24		1,300	12·1	0·14	0·07	0·54
25	3 gm. tyrosine	1,010	8·6	0·15	0·05	0·39
26		1,350	11·4	0·15	0·07	0·52
27		1,470	9·5	0·15	0·04	0·39
28	16 gm. casein	1,150	8·8	0·11	0·06	0·35
29		560	4·7	0·05	0·03	0·20
30		780	7·6	0·06	0·04	0·32
31	2·2 gm. asparagine	690	5·3	0·07	0·04	0·20
Aug. 1		1,090	8·7	0·08	0·06	0·38
2		1,010	9·4	0·10	0·06	0·39
3		630	5·5	0·05	0·04	0·27
4	14 gm. gelatine	1,040	5·2	0·09	0·04	0·25
5		610	7·3	0·13	0·07	0·44
6	16 gm. egg albumin	890	10·0	0·14	0·07	0·50
7 and 8 combined		1,010	8·3	0·09	0·05	0·36
9	16 gm. dried serum	1,250	8·6	0·09	0·06	0·34
10		880	7·3	0·07	0·04	0·38
11 and 12 combined		1,020	8·3	0·09	0·06	0·35
13		880	6·7	0·09	0·04	0·25
14	2·7 gm. cysteine hydrochloride	570	4·8	0·07	0·04	0·20
15 and 16 combined		2,350	19·7	0·18	0·15	0·82

TABLE 2.

Extra sulphur excreted after various amino acids and proteins.

Supplement	Date	Average N gm.	Extra sulphur excreted as						p.c. S.-containing amino acid recovered
			Cystine		Methionine		Sulphate		
			gm.	p.c.	gm.	p.c.	gm.	p.c.	
2 gm. cystine	July 19-20	9·7	0	0	0·07	13	0·48	89	102
2·5 gm. methionine	July 13-14	8·1	0·12	22	0·09	17	0·32	59	98
2·7 gm. cysteine hydrochloride	Aug. 15-16	9·9	0·03	5	0·11	20	0·13	24	49
2·5 gm. glycine	July 16-17	11·7	0·10		0·02		— 0·21		
2·2 gm. leucine	July 22-23	9·5	0·02		0·06		0·16		
3 gm. tyrosine	July 25-26	10·0	0·09		0·08		0·22		
2·2 gm. asparagine	July 31-Aug. 1	7·0	— 0·06		0·06		— 0·11		
16 gm. casein	July 28-29	6·8	— 0·05		0·04		— 0·14		
14 gm. gelatine	Aug. 4-5	6·3	0·01		0·07		0		
16 gm. egg albumin	Aug. 6-8	6·1	— 0·09		0·06		— 0·17		
16 gm. dried serum	Aug. 9-10	8·0	— 0·04		0·06		0·03		

TABLE 3.

Urinary sulphur excretion in relatives of the cystinuric boy.

	Volume	Total N (gm.)	Sulphur (gm.) as			Sulphur (mg.) excreted per gram N as		
			cystine	methionine	sulphate	cystine	methionine	sulphate
Father	1,940	11·8	0·05	0·06	0·60	4·1	5·1	51
Mother	2,330	14·8	0·05	0·08	1·02	3·4	5·8	61
Brother (24 years)	1,010	10·4	0·09	0·04	0·39	8·7	4·1	37
Brother (19 years)	1,520	9·6	0·04	0·08	0·57	4·0	6·5	60
Sister (16 years)	1,170	8·2	0·13	0·07	0·28	15·2	8·9	34
Brother (12 years)	1,520	10·4	0·04	0·08	0·63	3·7	7·6	61

The excretions of cystine, methionine and sulphate sulphur above the normal after the ingestion of the amino acid or protein supplement were calculated for the day on which the supplement was fed, and the succeeding day except in the following cases: (a) leucine: half a dose was given on each of two consecutive days, and increased excretion over that 48 hours' period only was used for the calculation. (b) egg albumin: as the second 24 hour specimen after the test dose was combined with the third, extra excretion was calculated over a period of 3 days. (c) cysteine hydrochloride: there was some doubt about the accuracy of the first 24 hour specimen obtained, so extra excretion was calculated for the period 24-72 hours after the test dose. The normal level of excretion for these calculations was considered to be the average excretion obtained for the first two days of the experimental period (July 11-12).

Despite an apparently consistent diet the total nitrogen excretion varied from day to day, a variation which bore no relation to the taking of protein or amino acid supplements. The results have therefore been expressed as the ratio of cystine, methionine and sulphate sulphur excreted per gram of nitrogen (Fig. 1). It was considered that this method of expressing the results would help to overcome discrepancies brought about by variations in protein metabolism.

The effect of single doses of the sulphur-containing amino acids.

Cystine. As with most cystinuric subjects, after the ingestion of cystine, there was no increase in cystinuria but a marked rise in urinary sulphate (in this case accounting for nearly 90 p.c. of the test dose). There was also a small rise in methionine excretion which could not be due entirely to the concurrent rise in nitrogen excretion (7·0 to 9·7 gm.) since the ratio of methionine to nitrogen excreted was also raised.

Methionine. Ingestion of equivalent amounts of methionine was also followed by a rise in urinary sulphate (though this was not as large as after cystine). There was a rise in methionine and also in cystine excretion whether expressed as total excretion or per gram of nitrogen. In respect this subject resembles the majority of those cases reported in the literature.

Cysteine Hydrochloride. Most cystinuric subjects have shown an increase in cystine excretion when cysteine hydrochloride has been given. Two exceptions have, however, been recorded (Hess and Sullivan, 1942; Cleland, 1946). Results obtained with this subject did not show any rise in urinary cystine, though there was a definite increase in methionine and sulphate. Nevertheless these increases in sulphur fractions only accounted for 44 p.c. of the ingested sulphur (as compared with 102 p.c. and 98 p.c. respectively after cystine and methionine). However, because there was doubt about the accuracy of the collection, results obtained for the 24 hours following the ingestion of cysteine hydrochloride were not used to calculate the increase in excretion. It is possible that during this period there was some increase in cystine excretion, particularly as the ratio of cystine to nitrogen excreted was raised in the specimen obtained on that day.

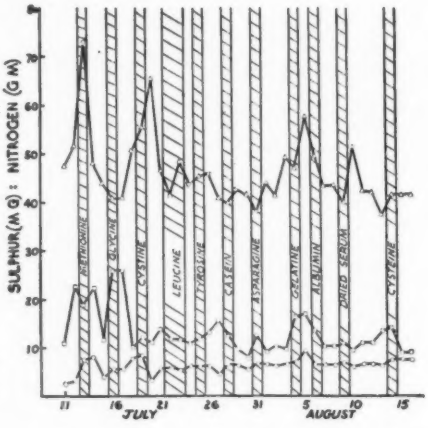

Fig. 1. Ratio of urinary sulphate, cystine and methionine sulphur to total urinary nitrogen excreted each day.

Sulphate sulphur (mg.): Total nitrogen (gm.).
Cystine sulphur (mg.): Total nitrogen (gm.).
Methionine sulphur (mg.): Total nitrogen (gm.).

The effect of single doses of the sulphur-free amino acids.

Because of the variable response to the ingestion of some amino acids obtained with other cystinuric subjects (Cleland, 1946, 1947), and because of the suggestion that cystinuria is associated with an abnormal renal threshold, for a number of amino acids (Dent, 1950), the effect of some sulphur-free acids on the excretion of cystine, methionine and sulphate sulphur was determined.

Glycine. In some cystinuric subjects glycine has been found to influence respectively nitrogen and sulphate excretion (Cleland, 1947) or methionine and sulphate excretion (Cleland, 1946) or (Hess and Sullivan, 1942) to increase cystine excretion. Interpretation in the last case is difficult since the concurrent nitrogen excretion was not recorded. In the present case the ingestion of glycine was followed by an increase in nitrogen and cystine excretion, but a fall in sulphate excretion. The increase in cystine was relatively greater than the increase in nitrogen.

Leucine. The consumption of leucine was followed by a small rise in the excretion of the sulphur-containing amino acids, but since there was an equivalent rise in nitrogen excretion, these increases were probably not significant. There was, however, quite a definite rise in urinary sulphate. It is concluded that leucine did not affect the degree of cystinuria in this case as it did in another (Cleland, 1947).

Tyrosine. The consumption of tyrosine was followed by an increase in all three sulphur fractions, but as excretion per gram of nitrogen was unaffected, it can be assumed that tyrosine did not affect the cystinuria.

Asparagine. There was a conspicuous fall in the excretion of cystine after taking asparagine, but this was offset by the lower nitrogen excretion. Indeed, although the total excretion of cystine was lower than normal, the cystine:nitrogen ratio was raised. However, these changes were not large and it was felt that asparagine did not bring about a significant alteration in the excretion of the three sulphur fractions studied.

The effect of various proteins.

If methionine and cysteine are the sources of urinary cystine in cystinuria, it would be expected that an increase in the amount of protein consumed or in the proportion of those proteins rich in methionine would be followed by an alteration in cystine excretion. Casein, egg albumin or dried serum, all good sources of methionine, when given in addition to the usual diet, did not affect the excretion of the various sulphur fractions even when allowance was made for the lower nitrogen excretion. There was, however, a delayed excretion of extra sulphate following the consumption of the dried serum. On the other hand equivalent amounts of gelatine (which contains only a trace of sulphur-containing amino acids) caused a relative rise in cystine excretion on the day of the test, and a delayed rise in sulphate excretion. It may be significant that the ingestion of glycine alone (the chief amino acid in gelatine) also produced a relative rise in extra cystine.

DISCUSSION.

It has always been difficult to reconcile the generally accepted theory that cystinuria is due to some inherent failure in the metabolism of cystine, with the ability of the cystinuric subject to metabolise dietary cystine normally. A lowered renal threshold for cystine, or an inability of the kidneys to re-adsorb cystine at the normal rate, could, however, explain this anomaly. Cystine is not very soluble and is probably only slowly absorbed from the intestinal tract, so that its ingestion would not cause a very great increase in blood cystine. The more soluble methionine and cysteine, however, are probably quite rapidly absorbed and readily converted into cystine, thus causing a rise in blood level and an increase in the amount appearing in the urine. Consumption of protein does not normally result in a very large increase in blood amino acid nitrogen, so presumably the blood cystine level is also little altered. This would explain why proteins rich in the sulphur-containing amino acids do not usually affect cystine excretion.

It has also been difficult to explain why metabolically unrelated amino acids should affect cystinuria. If, however, cystinuria is associated with an alteration in the renal threshold for a number of amino acids this could be explained by the relative effect of each amino acid on the re-absorption of the others. Thus, if the organism were flooded with one particular amino acid, it

is conceivable that this might interfere with the re-absorption of the others. In the present case it was noticeable that the only protein which affected cystine excretion was gelatine which is a readily soluble protein supplying large quantities of one particular amino acid, namely glycine.

If the renal threshold is an important factor in cystinuria, the following might influence the degree of cystinuria in any one subject:

i. The renal threshold for cystine. ii. The renal threshold for other amino acids. iii. The supply of cystine and cystine precursors. iv. The blood level of amino acids. v. The rate of absorption of amino acids during ingestion.

The results obtained with this patient could be explained by an abnormal renal threshold for cystine, the renal reabsorption of this amino acid being influenced by the presence in excess of other amino acids. With improvement in methods of amino acid analysis it should soon be possible to carry out amino acid tolerance curves and compare blood levels with concurrent urinary excretion. This would show whether cystinuria can still be considered as an inherent defect in cystine metabolism.

Two of the five siblings of this subject also showed an excessive excretion of cystine. The excretions of cystine, methionine and sulphate-sulphur and of nitrogen in single 24-hour specimens of urine from the patient's parents and siblings are recorded in Table 3.

SUMMARY.

Total nitrogen, and cystine, methionine and sulphate sulphur have been determined in the urine of a subject with cystinuria, his parents and siblings. The daily excretion of cystine by the subject was about 350–450 mg.

In the patient ingested cystine increased the urinary sulphate, caused a slight rise in methionine excretion, but did not affect the urinary cystine. The extra sulphur excreted accounted for 102 p.c. of the cystine given.

Ingested methionine increased the excretion of sulphate, methionine and cystine, the total increase in sulphur excreted accounting for 98 p.c. of the methionine.

Of ingested cysteine hydrochloride 44 p.c. was recovered as sulphate and though there was no absolute increase in cystine excretion there was a small rise in the proportion of cystine excreted per gram of nitrogen.

Leucine, asparagine or tyrosine did not affect significantly the excretion of the three sulphur fractions. The ingestion of glycine, however, was followed by a reduction in sulphate excretion and an increase in cystine excretion.

The addition to the diet of 16 gm. each of casein, egg albumin or dried serum was not followed by a rise in urinary nitrogen, or in an alteration in the rate of excretion of the sulphur-containing amino acids. The consumption of 14 gm. gelatine was, however, followed by a rise in urinary cystine.

These results are discussed with particular reference to the possibility that cystinuria is due to an altered renal threshold for cystine (and perhaps for other amino acids) rather than to some inherent abnormality in metabolism.

Acknowledgments. My thanks are due to my colleagues at the University of Adelaide, and at the Institute of Medical and Veterinary Science for help in preparation of the manuscript.

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MIXED BACTERIAL GROWTH

III. STUDIES ON THE MIXED GROWTHS OF A VIRIDANS STREPTOCOCCUS, A DIPHTHEROID AND *STAPHYLOCOCCUS ALBUS*

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In the first paper of this series (Annear and Hayes, 1950), a method for enumerating the viable organisms in mixed cultures of a viridans streptococcus and *Staphylococcus albus* was described and some mixed growth curves were studied. Media containing crystal violet and sulphanilamide were employed for obtaining selective recoveries of the streptococci and staphylococci. In a following paper (Hayes, 1950), further mixed growth curves of staphylococci and streptococci were presented; in this study, sodium chloride was used instead of sulphanilamide in the selective medium for staphylococci.

The present paper is concerned with experiments in which, by the same principles as those followed in previous studies, the population changes during pure and mixed growths of three organisms were followed. The organisms were a viridans streptococcus (Strain V2), *Staphylococcus albus* (Strain S6) and a diphtheroid (Strain D1). The streptococcus and the staphylococcus were the strains investigated in the first study in this series. The diphtheroid was isolated from a throat culture. For convenience, the code initials, V, S and D are used occasionally in this paper.

Crystal violet and sodium chloride were used for the selective recoveries of the streptococcus and staphylococcus. Anderson's medium (1944) with an increased tellurite concentration (1:750) was found to be suitable for the selective recovery of the diphtheroid.

In previous work, viable counts were made in agar media contained in Erlenmeyer flasks. Although this method was satisfactory, it did, when large numbers of counts were necessary, involve the use of a great deal of glassware and media. Preliminary experiments with the surface count method of Miles and Misra (1938) showed it to be suitable for these studies and to be simpler and more economical than the flask method. Consequently it was employed throughout this investigation.

Before mixed growth curves of the organisms could be studied, it was necessary to determine the relationships between known populations of the organisms and the agar media employed for enumerating them.

Only a summary of the results of these investigations is presented in this paper. The designs of the experiments were essentially the same as those described in detail in the first paper.

General Methods.

Media.

The nutrient broth, nutrient agar and diluent were of the same composition as described previously (Annear and Hayes, 1950).

Nutrient agar, though suitable for the recovery of the streptococcus and the staphylococcus, required enrichment before it was satisfactory for the recovery of the diphtheroid. Ward and Rudd's medium (1936) which was in constant use in this laboratory was found to be suitable for this latter purpose. This medium was also satisfactory for the recovery of the streptococcus and the staphylococcus but had no advantage over nutrient agar.

The compositions of the selective media are shown in Table 1.

Bacterial inocula.

As in previous studies, these were obtained from 20 hour broth cultures.

Viable counts.

As mentioned in the introduction to this paper, the method of Miles and Misra (1938) was used for making viable counts. In this method, the area of agar containing the colonies which arise from one drop of bacterial suspension is referred to as a 'drop area'. The number of these units from which mean counts were obtained varied from 2 to 6 and was usually not less than 3.

EXPERIMENTAL.

Organism-media relationships with static populations.

With 20 hour populations of organisms, experiments were carried out to determine the relationships between the recoveries of the organisms and the various selective and non-selective media employed. The results are summarized in Table 1.

TABLE 1.

Relationships between recoveries of organisms and various media.

Organism	Non-selective medium	Selective medium	Suppressed organisms
Streptococcus	Nutrient agar or * Blood agar	Nutrient agar + Crystal violet (1:1,000,000)	Staphylococcus: $1{\cdot}50 \times 10^6$ Diphtheroid: $1{\cdot}00 \times 10^5$
Staphylococcus	Nutrient agar or * Blood agar	Nutrient agar + Sodium chloride (10 p.c.)	Streptococcus: $5{\cdot}00 \times 10^6$ Diphtheroid: $1{\cdot}00 \times 10^5$
Diphtheroid	* Blood agar	Anderson's agar Pot. tellurite (1:750)	Streptococcus: $5{\cdot}00 \times 10^6$ Staphylococcus: $1{\cdot}50 \times 10^6$

* Ward and Rudd's agar.

The full interpretation of this Table may be exemplified by the following statement on the first row: Crystal violet agar suppressed the development of at least as many as $1{\cdot}50 \times 10^6$ staphylococci and/or $1{\cdot}00 \times 10^5$ diphtheroids and there were no significant differences between the streptococcal recoveries on crystal violet sown with these numbers of organisms, on crystal violet agar alone and on nutrient agar or blood agar.

In the first paper of this series experiments were described which tested the agreement between the counts resulting from the inoculation of the two organisms together in nutrient agar and the sum of those from the individual estimates. No attempt was made to check such agreement in this present work as the viable count method employed was unsuitable for it. Where mixed inocula of the organisms were colonising on the surface of agar, there was a tendency for the larger and faster growing staphylococcal colonies to engulf the neighbouring smaller and slower growing colonies of the streptococcus and the diphtheroid. With carefully selected inocula, or by increasing the area over which the drops spread, these effects may be obviated to some extent. However, the point was not pursued as the evidence presented in Table 1 is sufficient to justify summation of the selective agar counts to obtain total counts for the mixed populations.

Growth curves.

A growth curve experiment was carried out on lines similar to those of previous studies except that the organisms were grown in 150 ml. volumes of nutrient broth in 300 ml. Florence flasks.

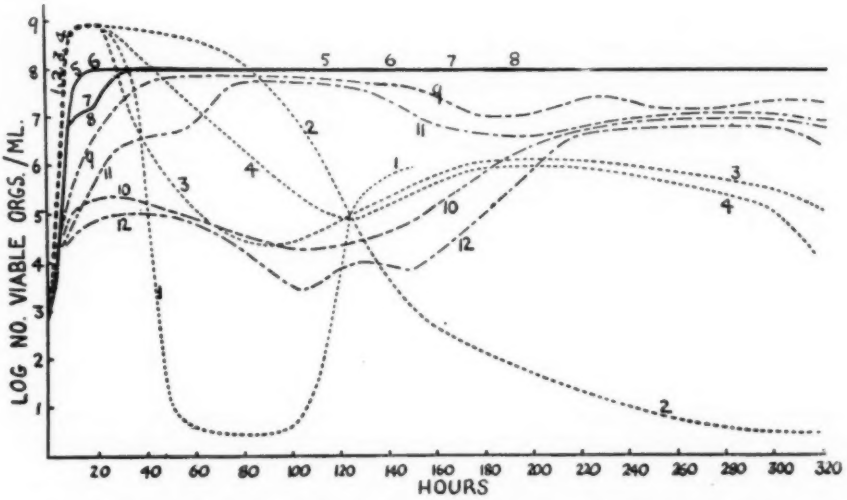

Fig. 1. Pure and mixed growths of a viridans streptococcus, *Staphylococcus albus* and a diphtheroid in nutrient broth at 37° C.

Streptococcus (V) – – – – – 1. pure. 2. in VD mixt. 3. in VS mixt. 4. in VDS mixt.
Staphylococcus (S) —— 5. pure. 6. in SD mixt. 7. in SV mixt. 8. in VDS mixt.
Diphtheroid (D) — — — 9. pure. 10. in DS mixt. 11. in DV mixt. 12. in VDS mixt.

Interpretation of the growth curves of the three organisms growing in association necessitated the parallel studies of the growths of the organisms in pairs and singly. The results of the experiments are shown in Table 2 and Fig. 1. The V and DS cultures showed contamination on the 7th and 20th days respectively and their study was accordingly discontinued. After the first day and with the exception of the 11th day, counts were made daily on the cultures for 13 days. Three further sets of counts were made on the 29th, 30th and 33rd days. The results of these latter counts are shown in Table 2 but have not been represented in Fig. 1.

TABLE 2.

Pure and mixed growths of a diphtheroid (D), Staphylococcus albus (S) and a viridans streptococcus (V) in nutrient broth at 37°C.

Hours	Log. number viable organisms/ml.											
	Diphtheroid				Staphylococcus				Streptococcus			
	Pure (D)	Mixed cultures			Pure (S)	Mixed cultures			Pure (V)	Mixed cultures		
		DS	DV	DSV		SD	SV	DSV		VD	VS	DSV
0	3·32	same inoculum as in Pure D			2·74	same inoculum as in Pure S			2·74	same inoculum as in Pure V		
3	4·02	3·88	3·78	4·02	3·74	3·81	4·04	4·13	4·70	4·74	4·48	4·81
6	4·81	4·54	4·78	4·70	5·18	5·26	5·45	5·65	6·54	6·78	6·38	6·74
9	N.C.	N.C.	N.C.	N.C.	6·98	7·15	6·88	6·74	8·65	8·81	8·67	8·98
12	5·95	5·33	5·10	4·81	7·93	7·88	7·10	7·13	8·93	8·90	8·98	8·74
27	7·20	5·40	6·32	4·90	7·90	7·90	7·90	7·85	8·60	8·90	8·40	8·78
55	7·95	4·90	6·74	4·85	8·26	8·24	8·19	8·29	—	8·70	5·43	7·48
77	7·90	4·60	7·88	4·42	8·32	8·24	8·24	8·27	—	8·16	4·54	6·48
103	7·88	4·30	7·65	3·48	8·24	8·22	8·28	8·08	—	6·74	4·45	5·48
128	7·70	4·56	7·78	4·10	8·06	8·06	8·18	8·00	5·11	4·54	5·02	5·00
150	7·65	4·85	7·06	3·85	8·00	8·02	8·32	7·84	6·00	3·04	5·48	5·32
175	7·11	5·81	6·74	4·88	7·85	7·88	8·28	7·98	cont.	2·30	6·02	5·95
200	7·13	6·48	6·65	6·19	8·11	7·93	8·15	8·00		1·70	6·11	6·04
224	7·48	6·81	6·90	7·02	8·18	8·00	8·22	7·95		—	6·02	6·02
244	7·28	7·00	7·04	6·74	8·19	8·13	8·33	7·93		—	5·90	5·70
296	7·35	7·32	7·13	6·85	8·16	7·81	8·30	8·19		—	5·47	5·11
320	7·27	6·88	6·93	6·40	8·37	7·74	8·39	8·26		—	5·11	3·95
696	5·95	cont.	*	7·29	6·74	cont.	7·18	5·81		—	—	—
720	5·70		*	7·13	7·19		7·18	5·74		—	—	—
792	6·27		*	5·70	7·38		6·85	6·02		—	—	—

* = colonies unsatisfactory for count. cont. = culture contaminated. N.C. = no count made. — = no colonies developed from 0·1 ml. of neat culture.

During the period between the 13th and 29th days, although no counts were made on the cultures, samples were plated out occasionally on the various media. These platings gave some indication of the relative levels of the organisms in the various cultures and it was because of an interesting trend that these platings revealed in the D S V association, that the last series of counts were made.

Streptococcal curves.

Comparison of the streptococcal curves revealed that the logarithmic phase of growth was not modified in the presence of the diphtheroid and/or the staphylococcus. The initial phase of decline, and the subsequent history of the streptococcus were, however, markedly modified in the presence of these two organisms.

The platings made after the 13th day showed that in the S V and D S V associations. the streptococci gradually died out until finally none was recovered. No reappearance of the streptococci was observed in these or in the D V association.

Staphylococcal curves.

Except for a period during the logarithmic phase, the growth of the staphylococcus was not significantly modified in the presence of the streptococcus and/or the diphtheroid during the first thirteen days of the experiment. Inhibition of the staphylococcus occurred during the

logarithmic phase in the S V and D S V associations but not in the S D association, indicating that in the D S V association, the effect was referable to the presence of the streptococcus. During this period in which the last three sets of counts were made, the staphylococcal level in the D S V association was significantly lower than in the pure culture.

Diphtheroidal curves.

The growth of the diphtheroid was markedly modified in the presence of the streptococcus and/or the staphylococcus. The inhibition of the diphtheroid in the D V association was of relatively short duration compared with its inhibition in the D S and D S V associations. These results indicate that the prolonged inhibition of the diphtheroid in the D S V association was referable to the presence of the staphylococcus. The last three readings revealed the diphtheroid level in the D S V association to be significantly higher than the pure culture level.

During the experiment continual check was made on the relationships between the recoveries of the organisms and the various agar media. It was found that as the staphylococcal cultures aged, the organisms became more sensitive to sodium chloride, and lower concentrations than 10 p.c. were necessary for full recoveries. No detailed investigation of this point was made but observations indicated that with all staphylococcal counts made during the experiment, there were no significant differences between recoveries on nutrient agar and on nutrient agar containing concentrations of sodium chloride as high as 7 p.c. The streptococcus was effectively suppressed by this concentration of salt but the diphtheroid, particularly in heavy concentrations, was not. Hayes (1950) found 5 p.c. sodium chloride satisfactory for studies on staphylococcal-streptococcal mixed growth curves. Confluent growth developed on 7 p.c. sodium chloride agar from massive inocula of the diphtheroid while smaller inocula were either completely suppressed or gave rise to a few colonies which developed very slowly. In the experiment recorded here, the concentrations of the diphtheroid in the mixed suspensions inoculated on the salt agar were never high enough to vitiate the staphylococcal counts on that medium.

Notice is drawn in Table 2 to the unsatisfactory diphtheroid recovery in the last three samples from the D V association. Apart from this finding for which no explanation can be given, and that with respect to the sodium chloride agar, there were no significant departures in the relationships between the colony growth and recovery media from those established with the static populations.

Biochemical observations.

A number of observations has been made which may help to explain some of the features of the pure and mixed growth curves. It has been recorded by several workers (e.g. Thompson and Johnson, 1947) that viridans streptococci may produce H_2O_2 during their growth in culture media. Other workers (e.g. Fuller and Maxted, 1939) have shown that organisms which produce H_2O_2 tend to die out very quickly and that in the presence of certain reducing substances or catalase, the death rate is retarded.

As the viridans streptococcus investigated in this present work exhibited a very rapid initial phase of decline when growing alone and moreover as this decline was markedly retarded in the presence of the staphylococcus and/or the diphtheroid, it was decided to examine the cultures concerned for H_2O_2 and catalase activity. Using the peroxidase test (Main and Shinn, 1941) and the titanium test (Bonet-Maury, 1944), positive reactions for H_2O_2 were obtained in cultures of the streptococcus while the staphylococcus and the diphtheroid yielded negative results. On the other hand, these two latter organisms showed strong catalase activity while the streptococcus showed none. It was also shown that the decline phase of the streptococcus was markedly retarded in the presence of lysed blood or cysteine. These findings are consistent with the hypothesis that the rapid decline of the streptococcus was due, in part at least, to H_2O_2 and that the retardation of the decline phase in the presence of the diphtheroid and/or staphylococcus was due to the catalase activity of the organisms.

It was at first thought that H_2O_2 might be the responsible factor in the inhibition of the staphylococcus and/or the diphtheroid which occurred in the presence of the streptococcus. It was shown, however, that lysed blood did not influence the inhibition, a finding which does not support a hypothesis involving H_2O_2 as the responsible factor for the effect.

DISCUSSION.

Evidence was presented in the first paper of this series to support the validity of the counts derived in the mixed growth curve experiments. Similar reasoning is applicable to this present work and will therefore not be repeated. No statistical data are presented in this paper; in many cases there were insufficient counts for worth-while analysis. There was, however, among the counts, no obvious evidence to invalidate the curves presented.

It was shown by Hayes (1950) that in nutrient broth, the viridans streptococcus after its initial phase of decline regenerates and undergoes a varying number of subsequent life cycles. This observation has now been confirmed many times in this laboratory. In the experiment reported here, contamination occurred during the second cycle. Secondary cycles of streptococcal growth are shown in the S V and D S V associations but not in the D V association. Failure of the streptococcus to regenerate in the D V associations is by no means a constant feature. In other experiments, not recorded here, regeneration occurred in much the same manner as shown in the other associations. There is, as yet, no evidence to explain these secondary growth cycles of the streptococcus.

Only tentative evidence has been presented for the rôle of H_2O_2 in the rapid decline of the streptococcus and for the catalase activity of the staphylococcus and diphtheroid as the means by which the decline of the streptococcus is retarded in the presence of these organisms. Similarly, it is by no means certain that H_2O_2 is inactive in the inhibition of the diphtheroid and/or the staphylococcus which occurs in the presence of the streptococcus. Careful quantitative study of the production of H_2O_2 by the streptococcus and of the sensitivities of the organisms to this metabolite are an essential part of the evidence required in these problems. In this respect it is noteworthy that the sensitivity among bacteria to H_2O_2 does not appear to be necessarily related to the catalase activities of the organisms (Molland, 1947).

No evidence has been accumulated to explain the prolonged inhibition of the diphtheroid which occurred in the presence of the staphylococcus.

So far in this work, emphasis has been placed on some of the significant differences between the growths of the organisms in pure and in mixed cultures. Another viewpoint from which the curves may be examined is that which has as its basis a comparison of the growths of the component organisms in particular mixed cultures. In this respect it is interesting to note that in the V S D association, each organism was for some time numerically dominant. Topley

and Fielden (1922) made similar observations on mixed cultures of faecal organisms.

No attempt is being made at present to discuss what might be regarded as minor trends and differences in the curves. Lucas (1949), in an excellent paper on ecological problems emphasized the diversity and complexity of external metabolites as they influence the interrelationships between living organisms and between living organisms and the inanimate world. It is almost certain that even in relatively simple systems such as those studied here there are a great many factors, both extrinsic and intrinsic, which influence the population changes of the organisms.

SUMMARY.

Three selective agar media containing crystal violet, sodium chloride and potassium tellurite were employed to enumerate the viable organisms in both static and dynamic mixed populations of a viridans streptococcus, *Staphylococcus albus* and a diphtheroid.

Some features associated with the mixed growths of these organisms are discussed.

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THE PHARMACOLOGY OF TRYPTAMINE

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Recently (Rapport, 1949) it has been shown that the vasoconstrictor substance which appears in serum incidental to the clotting process, is an indole derivative—probably 5-hydroxytryptamine. This discovery reawakens interest in tryptamine and its derivatives about which little information appears to be available (Laidlaw, 1911; Chen and Chen, 1933; Guggenheim, 1940).

This paper reports a study of the pharmacology of tryptamine. It deals with its circulatory and respiratory effects as well as its action on various test objects which have been used to study the pharmacological activity of serum.

METHODS.

Cats and dogs were anaesthetized with chloralose after preliminary ether anaesthesia; the dogs had also received an injection of morphine (10 mg./kg.). Rabbits were anaesthetized either with chloralose or urethane. The animals breathed naturally or were artificially ventilated. The systemic arterial blood pressure was recorded from the left carotid artery and the pressure in the right ventricle through a plastic catheter inserted *via* the right jugular vein. Sometimes, in the dog, the chest was opened and the pressure recorded in a branch of the pulmonary artery. All these pressures were recorded with mercury manometers. A saline manometer connected to a Brodie tambour was used to record pressures in the right auricle, the pulmonary vein and portal vein of the dog. The pressure in the latter vein was recorded through a plastic catheter inserted *via* the splenic vein after splenectomy. The action of tryptamine on the outflow of blood from the femoral vein in cats and dogs was examined by the method of Feldberg and Schilf (1930), the drug being injected either intravenously or through the cut stump of a branch of the femoral artery, the outflow being returned after measurement to a reservoir connected to the jugular vein. In dogs a Morawitz cannula was inserted into the coronary sinus after opening the thorax and the coronary flow measured. Heparin was used as an anticoagulant. To investigate its effect on the suprarenal gland tryptamine was injected both intravenously and into the cut stump of the coeliac artery in eviscerated cats in which the aorta was tied below the renal vessels (Feldberg and Minz, 1931).

In cats the respiratory movements were recorded by a tambour communicating with a pneumatic cuff applied around the thorax. The intrapleural pressure was recorded by a tambour connected through a water manometer with a plastic catheter which had been pushed through an intercostal space.

The right superior cervical ganglion was removed from a number of cats 7 or more days before the final experiment. Such animals were used, "intact" or after evisceration, to study

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the action of tryptamine on both the denervated and the innervated nictitating membrane. Arterial blood pressure was recorded from an iliac artery. The action of tryptamine on the suprarenal glands was also studied in some of these animals; in that case if both nictitating membranes were recorded it was necessary to record the blood pressure from an axillary artery.

Spiral strips of carotid artery from the ox or sheep, the jejunum of the guinea-pig and the uterus of the virgin rat were suspended in Ringer, Tyrode and anaphylactic Ringer solutions respectively, in isolated organ baths 10 ml. in volume, at 37° C.

The action of tryptamine injected intradermally in humans was also studied.

Throughout this paper doses of tryptamine are expressed in terms of the hydrochloride which was the preparation used.

RESULTS.

Circulatory effects in the dog.

The pressure in the carotid artery and in the right ventricle.

Following the intravenous injection of 1 to 8 mg. (0·1 to 1·0 mg./kg.) of tryptamine the first observable change is a rise of pressure in the right ventricle. As the pressure begins to rise the pulsations in the systemic manometer usually decrease in amplitude or the systemic arterial pressure may fall transiently. Some 5 to 10 seconds later the pressure in the carotid artery rises also; at this stage there is usually an increase in the amplitude of pulsation especially in the right ventricle and an increase in the heart rate. The rise of systemic arterial pressure is potentiated after division of the vagi. Both pressor responses last some 1 to 3 minutes and irrespective of whether or not the vagi are divided the rise of systemic arterial pressure is often followed by a fall lasting for a further 2 to 4 minutes. When similar injections of tryptamine were repeated at intervals of 10 minutes each rise of carotid pressure was equal in magnitude except in one experiment in which the second injection caused a greater systemic pressor response. On the other hand the rise of pressure in the right ventricle always became smaller and less well sustained with successive injections as is illustrated in Fig. 1. Respiratory effects in the dog were not examined; in any case they did not significantly influence the responses which were similar in pattern whether the animal breathed naturally or was artificially ventilated.

Fig. 1 shows the responses to three successive injections of 4 mg. of tryptamine into an 8 kg. dog with divided vagi under artificial respiration. It can be seen that pressure increase in the right ventricle which began 12 seconds after injection, rises at first abruptly and then more slowly; in the second phase of the rise the increased amplitude of pulsation becomes evident. The systemic arterial pressure began to rise 18 seconds after injection. The two stages in the rise of pressure in the lesser circulation can be seen very clearly in the experiment illustrated in Fig. 2, in which the thorax was opened and the pressure recorded in carotid artery, pulmonary artery, pulmonary vein and right auricle. At the arrow, 5 mg. of tryptamine were injected into the saphenous vein. During the first part of the rise in pressure in the pulmonary artery, the pressure in the pulmonary vein begins to fall, there is a decrease in the amplitude of the oscillations in the carotid manometer and a transitory rise of pressure in the right auricle. The rise of systemic arterial pressure begins at about the same time as the second phase of the rise of pressure in the pulmonary artery.

These experiments indicate that the rise of pressure in the pulmonary artery or right ventricle is due primarily to vascular constriction in the lungs and suggest that this accounts also for the initial changes in the systemic arterial pressure. A stimulating action on the heart as indicated by the increase in rate and amplitude of pulsation contributes to the remainder of the

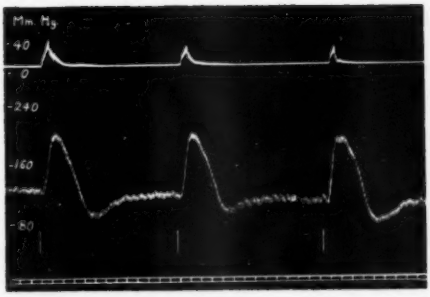
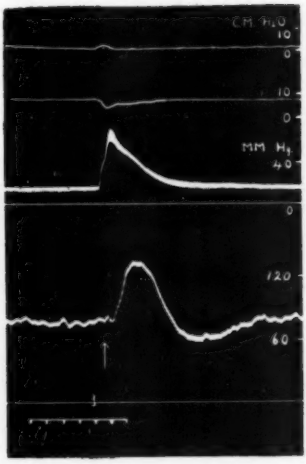


Fig. 1.



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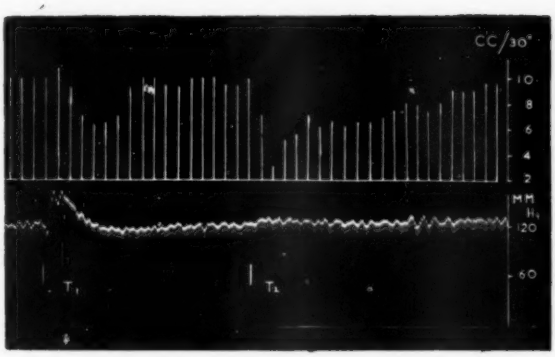

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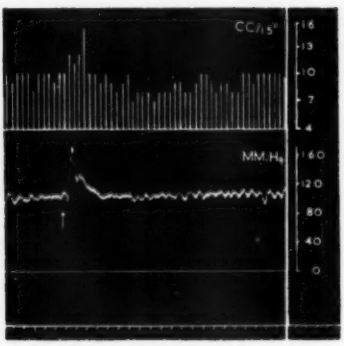

Fig. 4.

Fig. 1. Records of pressures in right ventricle (upper trace) and in carotid artery (lower trace) of a dog weighing 8 kg. with divided vagi, under artificial ventilation. At marks 4·0 mg. of tryptamine injected intravenously. Time in half minutes.

Fig. 2. Records (from above down) of pressures in right auricle, pulmonary vein, pulmonary artery and carotid artery of a dog weighing 8·4 kg. with divided vagi under artificial ventilation. At arrow 5 mg. of tryptamine were injected. Time in half minutes.

Fig. 3. Records from a dog weighing 9·0 kg. with intact vagi under artificial ventilation. The upper trace shows the outflow from the femoral vein in successive half minutes, represented as vertical lines ruled above the arterial pressure tracing, after varnishing. At T_1 7 mg. of tryptamine were injected intravenously and at T_2 1 mg. was injected intra-arterially. Details in text.

Fig. 4. Records from a dog weighing 19·0 kg. with intact vagi under artificial ventilation. The upper trace shows the outflow from the coronary sinus in each successive 15 seconds recorded as in Fig. 3. At the arrow 11 mg. of tryptamine were injected intravenously. Time in half minutes.

pressor response in the lesser circulation. Further experiments show that the rise of systemic arterial pressure is due partly to vasoconstriction and partly to cardiac stimulation.

The outflow from the hind limb.

When tryptamine in doses of from 0·1 to 1·0 mg. is injected into the femoral artery through the cut stump of a muscular branch, the arterial pressure is not significantly altered, and a diminished outflow from the femoral vein indicates therefore that vasoconstriction has occurred in the hind limb. When tryptamine is injected intravenously the blood flow from the femoral vein may increase transiently during the rise of arterial pressure after which it decreases, the reduction in flow outlasting the pressor response. Fig. 3 shows the arterial blood pressure and the outflow in each successive half-minute from the femoral vein of a 9·0 kg. dog with intact vagi, under artificial respiration. At T_1 7 mg. of tryptamine were injected intravenously, and at T_2 1·0 mg. was injected intra-arterially. In the same experiment 5 mg. given intravenously caused an initial fall of blood pressure. As the blood pressure fell the outflow from the hind limb fell also, and consideration of both the change in pressure and the outflow indicated that the initial depressor effect was not due to vasodilatation.

These experiments demonstrate a direct vasoconstrictor action of tryptamine. They show also that the depressor phases of the action of the drug are not due to vasodilatation arising reflexly or centrally. Further experiments in which tryptamine was injected into a carotid artery likewise disclosed no central action on the vessels of the hind limb.

The outflow from the coronary sinus.

The intravenous injection of tryptamine causes initially an increase in coronary flow while the heart increases in rate and appears to beat more vigorously. While the arterial pressure is still raised the coronary flow may return to its former level and remain there, or decline still further and continue at a reduced rate for some minutes. Fig. 4 shows the outflow from the coronary sinus of a 19 kg. dog with intact vagi, 11 mg. of tryptamine being injected at the arrow. The initial increase in flow can be explained by the direct action of tryptamine on cardiac muscle, the subsequent decline in flow by coronary vasoconstriction.

The pressure in the portal vein.

In view of the possibility that the depressor phases in the response of the systemic arterial pressure to the intravenous injection of tryptamine may be due to a constriction of the hepatic sphincter similar to that caused by histamine, the effect of tryptamine on the portal pressure was investigated. Following the intravenous injection of 2 to 8 mg. of tryptamine there is a rise of pressure in the portal vein which may be preceded by a transient fall; sometimes the portal pressure rose in two phases similar to that seen following the intravenous injection of adrenaline in the cat (McMichael, 1932). In any case when the same dose of tryptamine is injected directly into a mesenteric vein both the rise of arterial pressure and the rise of portal pressure are less than on systemic intravenous injection, showing that the rise of portal pressure is not due to an action in the liver.

Circulatory effects in the rabbit.

The rabbit is less sensitive to tryptamine than either the dog or the cat. Injections of 0·5 to 5·0 mg. (0·2 to 1·8 mg./kg.) cause a small rise in systemic arterial pressure which may be preceded by a transient fall and followed by a further fall lasting for two or three minutes. There is little change in the pressure in the right ventricle. Fig. 5, part 1, shows the pressure

in the carotid and in the right ventricle of a 3 kg. rabbit under urethane breathing naturally; at T_1 0·8 mg. of tryptamine was injected. Between the first two panels the vagi were divided. At S an injection of saline was made. The second part of the Figure shows similar records from a rabbit weighing 2·9 kg. under chloralose with intact vagi and artificially ventilated; at T_2 5 mg. were injected.

Circulatory and respiratory effects in the cat.

The pressure in the carotid artery and in the right ventricle.

Intravenous injections of 0·5 to 6·0 mg. (0·25 to 2·0 mg./kg.) cause a rise of pressure in the right ventricle followed in a few seconds by a rise of systemic arterial pressure. Whether or not the vagi are intact the rise of systemic pressure is sometimes preceded by transient fall

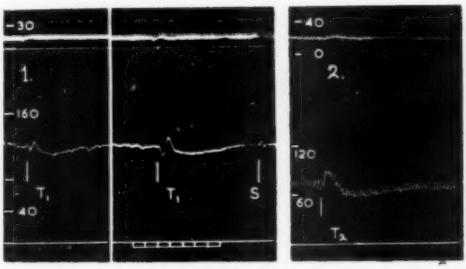

Fig. 5.

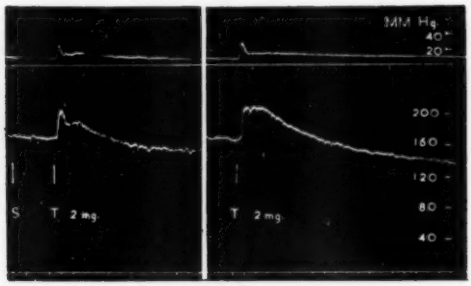

Fig. 6.

Fig. 5. Records as in Fig. 1. **Part 1.** Rabbit 3 kg. under urethane breathing naturally. At T 0·8 mg. of tryptamine was injected intravenously and at S 2 ml. of saline. Between the panels the vagi were divided. **Part 2.** Rabbit 3·2 kg. under chloralose; vagi cut, artificial ventilation. At T 5 mg. of tryptamine injected intravenously.

Fig. 6. Records (as in Fig. 1) of a cat weighing 3·3 kg. breathing naturally. Between panels vagi were divided. At T 2 mg. of tryptamine and at S 2 ml. of saline injected intravenously. Time in half minutes; interval between panels, 10 minutes.

similar to that illustrated for an eviscerated preparation in Fig. 8. Such an initial depression may not be due to pulmonary vasoconstriction because it occurred in one experiment in which the rise of right ventricular pressure was slight. During the early part of the rise of systemic arterial pressure the heart rate and the amplitude of cardiac pulsation in the manometers may increase. In animals breathing naturally there is also an increase in the amplitude of respiratory pulsation particularly in the right ventricular manometer and this may outlast the pressor response by several minutes (see Figs. 6 and 7). When the pressure in the right ventricle reaches its peak it may decline gradually to its initial level, or it may decline abruptly at first, followed either by a secondary rise as in Fig. 7 or by a further gradual fall to the initial level. After rising, the systemic arterial pressure may sometimes return to a level lower than before injection and may be unrecovered in 10 minutes. A second injection given after about 10 minutes causes an increment in systemic pressure which is usually equal in magnitude to the first one but sometimes it is less. A greater pressor response to tryptamine usually follows division of the vagus nerves.

Fig. 6 shows the pressure in the right ventricle and in the carotid artery of a cat weighing 3·3 kg. breathing naturally. At S 2 ml. of saline and T 2 mg. of tryptamine were injected

intravenously. Between the two panels is an interval of 10 minutes during which the vagus nerves were divided.

After the intravenous injection of tryptamine generalised muscular twitching lasting for a few seconds was often seen. Frequently the bladder was evacuated within the few minutes following an injection.

Respiratory effects.

Laidlaw (1911) described respiratory failure following the injection of 5 mg. of tryptamine into cats anaesthetized with chloroform or ether; much larger doses were required in unanaesthetized cats. In the experiments described above, respiratory effects did not appear to contribute significantly to the vascular responses obtained. The only difference between the responses of naturally breathing animals and those artificially ventilated was the increased amplitude of the respiratory pulsations in the former. To examine more closely the action of tryptamine on respiration, the respiratory movements and the pressure in the intrapleural space were recorded as well as the pressure in the carotid artery and right ventricle.

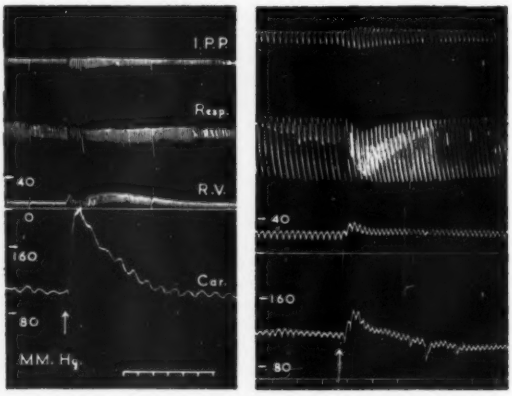

Fig. 7. Records (from above downwards) of intrapleural pressure, respiratory movements, pressure in right ventricle, pressure in the carotid artery and time in half minutes. First panel: cat 3·5 kg., vagi intact. At arrow 5 mg. of tryptamine injected. Second panel: cat 3·9 kg. with divided vagi. At the arrow 3 mg. of tryptamine given intravenously.

Fig. 7 illustrates two such experiments. In each panel a downward movement in the upper two tracings denotes inspiration. The first part of Fig. 7 is taken from an experiment in which 5 mg. of tryptamine were injected intravenously (at the arrow) into a 3·5 kg. cat with intact vagi. As the pressure in the right ventricle begins to rise there is a period of apnoea lasting for the duration of two or three respiratory cycles. For a short period after the apnoea respirations are irregular and shallower in depth, and the expiratory volume of the chest is somewhat increased. Despite the shallowness of respiration the amplitude of the change in intrapleural pressure is increased and this continues after the depth of respiration has returned to normal. As the amplitude of the intrapleural pressure increases so does the amplitude of the respiratory rhythm in the blood pressure tracings, particularly that of the right ventricle. In this experiment it is so increased that the mean right ventricular pressure falls for a short time below atmospheric pressure, during inspiration.

The experiment of the first panel of Fig. 7 illustrates, therefore, a depressing action on respiration and bronchoconstriction. Sometimes the record of respiratory movement is interrupted by muscular twitching occurring within a few seconds after injection: sometimes there is no period of apnoea, respiration being simply decreased in depth and rate for about half to one minute.

When apnoea followed a first injection of tryptamine it occurred also after a second or a third. In two experiments division of the vagi prevented the short period of apnoea which had been observed before their division but it did not prevent the bronchoconstriction as evidenced

by the change in intrapleural pressure and the increase in the expiratory volume of the chest. The second panel of Fig. 7 shows the responses in a cat weighing 3·9 kg. with divided vagi. An injection of 3 mg. of tryptamine at the arrow caused an increase in the expiratory volume of the chest and an increase of the amplitude of the intrapleural pressure change.

The outflow from the hind limb.

The injection of tryptamine into the arterial supply of the hind limb of the cat causes a decrease in outflow from the femoral vein. The constrictor substance in cat serum causes in this type of preparation a similar diminution in outflow (Reid, 1947). In one experiment an outflow of 24 drops per half minute was just measurably reduced by 5 μg. of tryptamine; 10 μg. reduced it to 19 drops per half minute, recovery occurring in 4 minutes; 0·5 ml. of serum prepared from the same cat reduced it to 16 drops per half minute; recovery occurring in 6 minutes and 20 μg. of tryptamine reduced it to 11 drops, recovery occurring in 8 minutes. When tryptamine is injected intravenously the outflow from the femoral vein is reduced even during the rise of arterial blood pressure and the reduced outflow continues for some minutes after the pressor response.

Action in the eviscerated cat and on the suprarenal medulla.

A rise of systemic arterial blood pressure is regularly seen in the eviscerated preparation after the intravenous injection of tryptamine in doses ranging from 0·05 to 1·0 mg. The rise may be preceded, as in the intact animal, by a transient depression. When tryptamine is injected into the cut stump of the coeliac artery in doses up to 1 mg. the blood pressure does not change or it rises after a longer latency and is considerably less in magnitude than when injections are made intravenously. Likewise when the response of a denervated nictitating membrane is recorded also, contraction resulting from the administration of tryptamine is regularly less when it is injected into the vascular supply of the suprarenal glands than when it is injected intravenously.

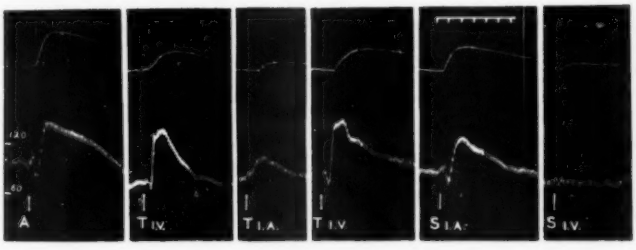

Fig. 8.

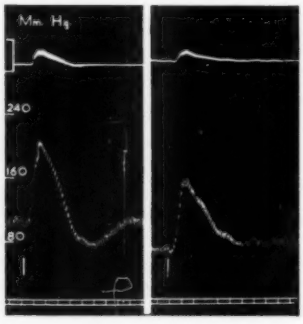

Fig. 9.

Fig. 8. Records of a denervated nictitating membrane and arterial blood pressure of an eviscerated cat weighing 2·8 kg. under artificial respiration and with divided vagi. At A, 4 μg. of adrenaline were injected intravenously. At T 0·5 mg. of tryptamine and at S 0·5 ml. of cat serum were injected, the letters I.V. and I.A. denoting whether injections were made intravenously or into the coeliac artery respectively.

Fig. 9. Records (as in Fig. 1) from a dog weighing 8 kg. with divided vagi under artificial ventilation. At the marks 4 mg. of tryptamine were injected and between the panels 2 mg./kg. of yohimbine. Time in half minutes.

Fig. 8 shows the pressure in the left carotid artery of a cat with divided vagi under chloralose anaesthesia (lower tracing) and the response of the right nictitating membrane which had been denervated 10 days previously. The responses are to 4 μg. of adrenaline hydrochloride injected intravenously at A, to 1 mg. of tryptamine at T, the letters I.V. or I.A. denoting whether injection was made intravenously, or intra-arterially into the cut stump of the coeliac artery respectively. It can be seen that the response of both arterial pressure and the nictitating membrane is greater following the intravenous injection. On the other hand, 1 ml. of the cat's own serum, which stimulates the suprarenal medulla (Reid, 1948) caused a greater rise of pressure and a greater contraction of the nictitating membrane when it was given intra-arterially (at S. I.A.) than when given intravenously (at S. I.V.).

Action in the renal circulation.

In three experiments the action of tryptamine on the renal blood vessels was investigated. Two of the cats were eviscerated, the aorta and inferior vena cava tied below the renal vessels and the suprarenal veins ligated. A reservoir and cannula were connected to a jugular vein and the animal was heparinized. A cannula directed cranially was inserted into the inferior vena cava which was then tied a second time, above the renal veins. The blood from this part of the cava consisting mainly of renal blood was returned through the cannula to the jugular reservoir. The intravenous injection of 1 to 3 mg. of tryptamine caused the blood pressure to rise and the outflow from the kidney to increase. The injection of up to 5 mg. of tryptamine into the cut stump of the superior mesenteric artery caused a slight transient decrease in the renal flow. Finally at the height of a tryptamine response Indian ink injected intra-arterially filled both cortex and medulla. In the third experiment the outflows from the hind limb and from the left renal vein were measured simultaneously. During the rise of blood pressure following the intravenous injection of 5 mg. of tryptamine the renal blood flow rose while the hind limb blood flow fell.

The influence of other drugs on the vascular effects of tryptamine

Yohimbine in doses up to 2 mg. per kg. which is more than adequate to reverse the action of adrenaline, reduced but did not abolish the pressor responses in both carotid artery and right ventricle of the cat and the dog. An experiment illustrating this is shown in Fig. 9 which represents records from a dog weighing 8 kg. with divided vagi, under artificial ventilation. The first response in the Figure is the fourth one in this particular animal to 4 mg. of tryptamine. Between the two panels 2 mg. per kg. of yohimbine were injected and 8 minutes later, in the second panel, 4 mg. of tryptamine were again injected. Yohimbine also reduced the vasoconstrictor action of tryptamine in the hind limb. Thus in one cat the intra-arterial injection of 50 μg. had a greater action before yohimbine than 100 μg. after it.

The influence of cocaine and benadryl were observed only in the eviscerated cat. No potentiation by cocaine was observed. Benadryl reduced somewhat the pressor response to tryptamine (see Fig. 12).

The nictitating membrane of the cat.

Since the results disclosed that tryptamine did not release active substances from the suprarenal gland some of these experiments were carried out in animals with intact suprarenals. In others in which the action of serum was studied as well, the suprarenals were removed at the beginning of the experiment. The intravenous injection of tryptamine in doses ranging from 0·05 to 1 mg. (0·01 to 0·5 mg./kg.), caused contraction of the denervated nictitating membrane. In many experiments the response of normal (left) membrane was recorded also, the left superior cervical ganglion being removed at the beginning of the experiment. In seven

experiments the threshold of the denervated membrane was clearly lower than that of the normal one, and over a wide range of dosage the response of the former was greater than that of the latter (Figs. 10, 11, and 12). Fig. 10 shows the response of both denervated and innervated membranes in an eviscerated adrenalectomized cat with divided vagi under artificial respiration. The denervated membrane shows some spontaneous rhythmic activity. At A 4 μg. of adrenaline, at T_1 and T_2 50 and 500 μg. respectively of tryptamine and at S 1 ml. of the cat's own serum were injected intravenously. The first portion of the tracing shows that the denervated membrane is more sensitive to tryptamine and serum than is the innervated membrane. Since the animal was adrenalectomized the action of serum on the membrane must be other than through the liberation of adrenaline, extending the observation of Zucker (1944), who injected serum *via* the carotid artery. In some experiments I injected tryptamine or serum distally into a carotid artery and in each case the response of the nictitating membrane was greater than when injections were made intravenously.

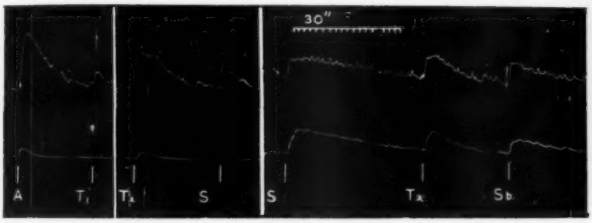

Fig. 10.

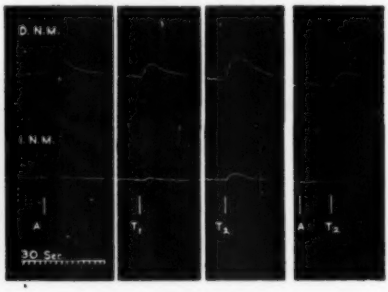

Fig. 11.

Fig. 10. Records of denervated (upper trace) and innervated (lower trace) nictitating membrane of an eviscerated adrenalectomized cat with vagi cut under artificial respiration. At A 4 μg. of adrenaline at T_1 and T_2 50 and 500 μg. respectively of tryptamine, at S 1 ml. of cat serum and at Sb 0·5 ml. of boiled cat serum were injected intravenously. Between the last two panels cocaine, 6 mg., was injected. Time in half minutes.

Fig. 11. Records (as in Fig. 10) of a 1·5 kg. cat breathing naturally with vagi intact. Adrenaline 2 μg. tryptamine 0·4 and 0·8 mg. were injected intravenously at A, T_1 and T_2 respectively. Between the last two panels 1·5 mg. of yohimbine was injected.

Influence of cocaine.

The second part of Fig. 10 shows how cocaine may potentiate the response of the innervated membrane to both serum and tryptamine. Between the last two panels 6 mg. of cocaine were injected intravenously. In this part of the Figure 1 ml. of serum was given at S, 500 μg. of tryptamine at T_2 and 0·5 ml. of serum which had been boiled at Sb. When the cocaine itself caused a great increase in tone of the innervated membrane no potentiation of the effect of tryptamine or serum was observed. These results recall those of Zucker (1944) who showed that cocaine potentiated the effect on the innervated nictitating membrane of a muscle-stimulating substance prepared from blood platelets.

Influence of yohimbine.

Yohimbine reduced the response of both innervated and denervated nictitating membranes to tryptamine but not to the same degree as it reduced comparable responses due to adrenaline. Fig. 11 shows the responses of both innervated and denervated membranes of a cat breathing

naturally with intact vagi to 2 μg. of adrenaline at A and to 0·4 and 0·8 mg. of tryptamine at T_1 and T_2 respectively. Between the last two panels 1 mg. per kg. of yohimbine was injected intravenously after which artificial respiration had to be commenced.

Influence of benadryl.

This drug usually caused a slow sustained contraction of the denervated membrane and depressed its response to tryptamine. It potentiated the response of the innervated membrane to both tryptamine and serum. Fig. 12 shows the responses of both nictitating membranes and the pressure in an axillary artery of an eviscerated cat to 0·2 mg. of tryptamine injected intravenously at the strokes. Between the first two panels 5 mg. of benadryl was injected.

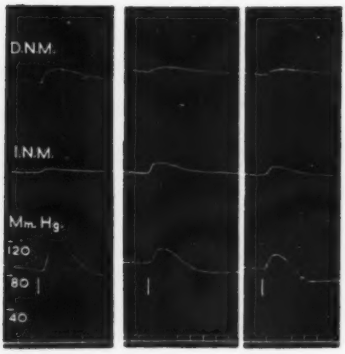

Fig. 12.

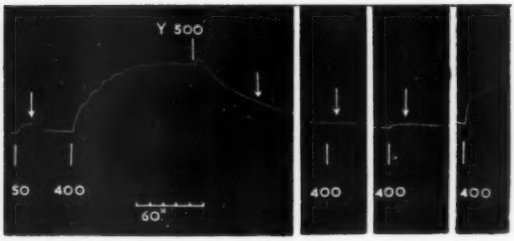

Fig. 13.

Fig. 12. Upper two traces as in Fig. 10; lower trace is record of the pressure in an axillary artery of an eviscerated cat with divided vagi. 0·2 mg. of tryptamine was injected at the strokes. Between the first two panels 5 mg. of benadryl were injected. Time in half minutes.

Fig. 13. Record of spiral strip of sheep carotid artery. Injections were made at the strokes and at the arrows the bath was washed out. The figures denote μg. Time in minutes. Intervals between the panels 8, 10 and 120 minutes respectively.

Other preparations.

The pupil of the cat.

The action of tryptamine on the cat's pupil is similar to that of serum. The intravenous injection of 0·2 to 0·8 mg. of tryptamine causes an initial dilatation (more marked on the denervated side) followed by a strong constriction which lasts about as long as the contraction of the nictitating membrane. Sometimes the pupil became irregularly circular or asymmetrical in shape. These observations confirm those of Laidlaw although he states that he did not observe them in anaesthetized animals. They show also that serum has a similar action confirming the observations of Zucker (1944).

The arterial strip of sheep or ox.

Tryptamine causes contraction of the isolated spiral strip of carotid artery after a latency of 1 to 10 seconds. The threshold dose varied from about 2 μg. to 20 μg. Fig. 13 shows the response of a carotid artery of the sheep to tryptamine injected at the strokes, the figures

denoting μg. At Y 500 μg. of yohimbine hydrochloride caused relaxation of the contraction due to tryptamine. After washing out the bath at the second arrow and allowing the tracing to return to its original level tryptamine was without action and the preparation had not fully recovered after more than 2 hours.

The uterus of the virgin rat.

Tryptamine causes an increase in tone and in the amplitude of the spontaneous rhythm. When the rhythm is poor its increase may be the sole effect of a small dose. In various preparations the threshold varied from 0·5 μg. to about 20 μg. Usually the magnitude of the responses decreases with successive doses. On washing out the bath after 1 or 2 minutes the spontaneous rhythm may be potentiated or after a brief period of potentiation may be depressed. A second injection of tryptamine during such a depression potentiates the rhythm once more. Fig. 14 shows the response of a horn of a uterus to 50 μg. of tryptamine at T_1; the second response shown is much smaller than the first. At S_1 and S_2 0·05 and 0·1 ml. respectively of cat serum was injected and between them, at T_2, 40 μg. of tryptamine. The last portion of the Figure shows the response of a horn of a uterus from another rat to 2 μg. of tryptamine.

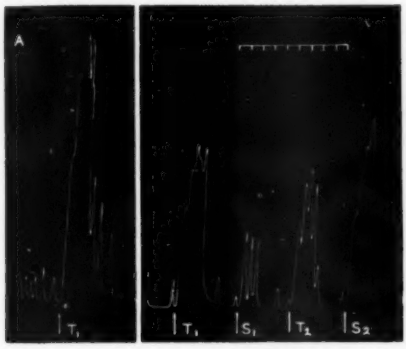

Fig. 14.

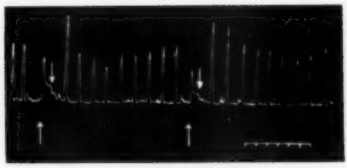

Fig. 15.

Fig. 14 Record of contraction of the isolated virgin uterus from two rats. At T_1 50 μg., at T_2 40 μg. and at T_3 (in a second uterus) 2 μg. of tryptamine were injected. At S_1 and S_2 0·05 and 0·1 ml. of cat serum respectively were injected. Time in minutes. Interval between first two panels 43 minutes.

Fig. 15. Isolated jejunum of guinea-pig. At ↑ 50 μg. of tryptamine were injected and at ↓ the bath was washed out. The unlettered responses are to 0·2 μg. of histamine. Time in minutes.

Yohimbine in doses of 20 to 80 μg. reduced or abolished the response to tryptamine. When administered during a tryptamine contraction it relaxed the uterus and abolished its rhythm, the contraction and rhythm returning when the bath was washed out and tryptamine added again.

The jejunum of the guinea-pig.

The jejunum of the guinea-pig is relatively insensitive to tryptamine. The gut responds after a short latency varying from 1 to 7 seconds and successive doses at intervals of 10 minutes or less give decreasing responses. For example in one experiment 500 μg. caused a smaller res-

ponse than that given originally by 50 μg. while the responses to histamine or cat serum were scarcely altered. Thus in the same experiment 0·1 ml. of the serum was in the beginning equivalent to 50 μg. of tryptamine and in the end, equivalent to more than 500 μg. When the bath is washed out 30 to 60 seconds after the addition of tryptamine relaxation is usually rapid, sometimes interrupted by a shortlived spontaneous rhythm. Fig. 15 shows the effect of the first two injections of 50 μg. of tryptamine; the unlettered responses are to 0·2 μg. of histamine acid phosphate. After washing out the responses to histamine are at first potentiated and then depressed before returning to the initial level. These after-effects were common; sometimes the potentiation only, sometimes the depression only was observed.

The vessels of the human skin.

The intradermal injection in ten subjects of tryptamine hydrochloride (B.D.H.) in concentrations up to 1/200 caused no blanching. Usually there was a slightly greater red reaction around the site of injection than was given by saline alone and this persisted for half to three quarters of an hour.

DISCUSSION.

Laidlaw (1911) showed that tryptamine raised the systemic arterial blood pressure of the cat. These results show that it has a pressor action in the systemic and pulmonary circulations of the cat and dog and similar but slighter effects in the rabbit. Its action in the rabbit has not been explored; it is known to have a vasoconstrictor action on the rabbit's ear (Rapport, Green and Page, 1948) and Laidlaw (1911) demonstrated a dynamic action on the rabbit's heart perfused by Langendorf's method. The systemic pressor response in the cat and dog is due partly to an action in the heart and partly to vasoconstriction. Laidlaw's experiments on the isolated rabbit's heart indicate a direct action of tryptamine on cardiac muscle and its failure to liberate active substances from the suprarenal glands of the cat indicates that there can be no indirect action mediated by such means. Laidlaw observed vasoconstriction in the viscera of the cat; my results show that tryptamine has a direct action on the vessels of the hind limb of the dog and cat and on isolated arterial strips but it does not appear to constrict the vessels of the human skin even in large doses. The latter result is surprising in view of Laidlaw's observation that tryptamine causes blanching of highly vascular surfaces although he does state that it is not nearly so effective as adrenaline. Curiously, the action of tryptamine on the renal circulation appeared weak or absent in comparison with its action in the hind limb.

Tryptamine also has a vasoconstrictor action in the pulmonary and coronary circulations; in the latter situation the vasoconstriction may be obscured by the dynamic effect on the heart. Its action in these vascular fields may modify an otherwise simple systemic pressor response. In the dog sometimes there is an initial fall of systemic pressure and its association with a rise of pressure in the pulmonary artery and right auricle and a fall of pressure in the pulmonary vein strongly suggests that it is due to pulmonary vasoconstriction. Furthermore, there is no evidence of peripheral vasodilation or a direct

or reflex cardiac action to account for it. So far as the secondary fall of systemic arterial pressure in the dog is concerned the results indicate that it is not due to pulmonary vasoconstriction, to reflex action on the heart through the vagus, nor to peripheral dilatation of central, reflex or direct origin. It is not due to constriction of the hepatic sphincter; indeed, the action of tryptamine on the pressure in the portal vein is not due to an action in the liver itself, and is probably a passive result of the rise of arterial blood pressure. The late reduction in coronary flow suggests that the secondary fall of blood pressure in the dog may be cardiac in origin but there is no direct evidence that this is the case. It is supported, however, by the fact that occasionally the pressure in the lesser circulation also may fall transiently before returning to normal.

In the cat the origin of the occasional depressor phases of the response to tryptamine has not been satisfactorily elucidated. No evidence of a vasodilator action in the hind limbs was observed following either the intravenous or the intra-arterial injection of tryptamine which indicates that there is no central, reflex, or direct vasodilator action. This makes it likely that a diminished output of the left ventricle is responsible. It is possible that pulmonary vasoconstriction, coronary constriction and vagal inhibition all contribute to this, the significance of each varying from one cat to another. Thus the initial depression of blood pressure occurs in animals with divided vagi so that vagal inhibition cannot be the sole factor. On the other hand, in one experiment in which the vagi were intact the initial fall of systemic arterial pressure was unassociated with a great rise of pressure in the pulmonary artery, so that in this experiment, at least, it was not due to pulmonary vasoconstriction. In any case the initial fall of blood pressure is observed inconstantly not only from one cat to another but from one injection to another in the same animal; this makes its nature difficult to determine.

The brief period of apnoea in the cat is not a reflex result of the raised systemic arterial pressure because it begins before the rise of pressure. Laidlaw (1911), who described more severe respiratory depression than I have observed regarded it as central in origin. While these results do not definitely exclude a central action they suggest that the apnoea arises from stimulation of vagal endings. Respiratory and cardiac effects in the cat are known to arise reflexly from vagal stimulation by a number of other substances including A.T.P. (Emmelin and Feldberg, 1948), a toxic protein in serum (Brodie, 1900) and veratrine (Dawes, 1947).

Whatever may be the nature of the respiratory depression it is abundantly clear that the circulatory responses and the contraction of the nictitating membrane are not simply due to the liberation of adrenaline or other reflex result of such depression. Thus the nictitating membrane and the blood pressure respond similarly whether respiration is artificial or natural; they respond in

adrenalectomized animals, and both the blood vessels of the hind limb and the nictitating membrane respond to the intra-arterial injection into the appropriate field of doses too small to influence respiration.

Because serum may contain several active substances undue emphasis must not be placed on similarities between its actions and those of tryptamine. Nevertheless, it is interesting that cat serum and tryptamine have similar effects on the hind limb, the nictitating membrane and the pupil of the cat, the carotid artery and the rat uterus; and that in all these preparations their pharmacological potentiations and antagonisms are similar. Thus the vasoconstrictor action of serum (Heymanns *et al.*, 1932; Bayliss and Ogden, 1933; Bing, 1941) and its action on vascular strips (Reid, 1946) are antagonized by yohimbine or ergotoxine. These experiments show that the pressor action, the vasoconstrictor action and the contraction of the arterial strip by tryptamine are also antagonized by yohimbine. Laidlaw (1911) observed that the pressor response to tryptamine is antagonized by ergotoxine. The response of the nictitating membrane to both serum and tryptamine, is potentiated by denervation and decreased by yohimbine. When the response of the innervated membrane to tryptamine is potentiated by cocaine or benadryl so also is the response to serum. These results are consistent with the view that the vasoconstrictor substance of serum is a tryptamine derivative (Rapport, 1949) and is identical with the substance stimulating the nictitating membrane and the uterus of the rat. They indicate that it is unlikely to be tryptamine itself because in that case serum would need to contain relatively large quantities to account for its action on the rat uterus, carotid artery and nictitating membrane. Thus in the experiment of Fig. 10, 1 ml. of cat serum was equivalent to more than 0·5 mg. of tryptamine. Moreover, the same Figure shows that the potentiating action of cocaine is greater for serum than for tryptamine.

The jejunum of the guinea-pig becomes less sensitive to repeated doses of tryptamine but not to serum. The after-effects of an injection of tryptamine on the response to histamine are similar to those of serum (Reid, 1946) but these after-effects are seen to follow contraction of the jejunum by other substances such as S.R.S. (Feldberg, Holden and Kellaway, 1938). There is nothing in these experiments therefore to suggest that the gut-stimulating substance in serum is a tryptamine derivative. So far as the substance in serum which stimulates the suprarenal medulla is concerned these results likewise give no indication that it is a tryptamine derivative. It is therefore not identical with the serum vasoconstrictor, if the latter is in fact a tryptamine derivative, unless such a derivative has an action on the suprarenal glands not shared by tryptamine itself.[2]

[2] Since this paper was submitted, I have found that the purified serum vasoconstrictor has actions on the guinea-pig gut similar to tryptamine. It also stimulates the suprarenal gland.

SUMMARY.

In the cat and the dog and the rabbit tryptamine causes a rise of systemic arterial pressure which is occasionally preceded or followed by a fall. These effects can be seen when the vagus nerves are intact or divided. In the dog and cat and to a slighter degree in the rabbit there is also a rise of pressure in the pulmonary artery or right ventricle. The pressor response in each circulation is reduced by yohimbine.

The rise of systemic arterial pressure in the cat and dog is due to a direct vasoconstrictor action (demonstrated in the hind limb) and to a dynamic effect on the heart. In the dog the initial fall is due to pulmonary vasoconstriction and the secondary fall possibly to coronary vasoconstriction. The outflow from the coronary sinus in the dog is increased during the dynamic action on the heart following which the outflow may decrease for some minutes. The action of tryptamine on the kidney (cat) is weak or absent compared with its effect in the hind limb.

The portal pressure in the dog is raised by the systemic intravenous injection of tryptamine. This is not due to constriction of the hepatic sphincter but appears to be a passive result of the raised arterial pressure.

The drug causes a brief period of apnoea in the cat which is not observed after vagal division. It also causes bronchoconstriction. Muscular twitching and evacuation of the bladder were also observed in the cat.

When injected into the arterial supply of the suprarenal glands tryptamine does not liberate adrenaline.

It contracts the nictitating membrane of the cat, this action and the similar action of cat serum being sensitized by denervation and depressed by yohimbine. The response of the innervated membrane is sometimes sensitized to both serum and tryptamine, by benadryl or cocaine. The pupil of the cat dilates transiently and then constricts strongly to tryptamine and serum.

Th drug stimulates isolated arterial strips, the rat uterus and the guinea-pig intestine.

It does not cause blanching of the human skin when injected intradermally.

These actions of tryptamine are discussed in relation to the view that the vasoconstrictor substance of serum is a tryptamine derivative.

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DEAMINATION OF ASPARTIC ACID BY *PROTEUS* X-19.

I. STIMULATION OF ANAEROBIC DEAMINATION BY GLUCOSE, ADENOSINE AND GLUTATHIONE

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Many bacteria are known to deaminate aspartic acid anaerobically, by the so-called desaturation reaction, to yield fumaric acid and ammonia. In 1938, Gale demonstrated in *E. coli* the existence of such an enzyme, differing from that previously studied in being inhibited by toluene and stimulated by adenosine and certain other substances. A few references have been made to this system in succeeding years (for example Lichstein and Christman, 1948; Axelrod *et al.*, 1948), but little work has been carried out on the mechanism by which these stimulatory materials exert their effect.

The following is an account of the aspartic deaminase system in *Proteus* X-19 and the effect of fermentation, purine derivatives and reducing substances thereon.

Materials and Methods.

Organism. The results given below were obtained with a strain of *Proteus* X-19 from the Commonwealth Serum Laboratories, Melbourne. The system has been shown to be similar in two other strains, one isolated locally by the Bacteriology Department, University of Adelaide and the other obtained originally from the Lister Institute.

The organisms were grown on nutrient agar, or on heart infusion agar (Difco) of pH 7·6 and after incubation for 15 hours at 37°C were harvested in distilled water, washed twice and made up in the appropriate buffer.

Dry weight was determined by drying an aliquot overnight *in vacuo* over P_2O_5.

l-Aspartic acid. This was prepared from l-asparagine by the method of Schiff (1844) and had an optical rotation of $(\alpha)\ \frac{20}{D} = +25°$ (0·1 M in N HCl).

Ammonia was estimated by distillation into alcoholic boric acid in Conway units followed by titration with hydrochloric acid (Conway and O'Malley, 1942).

Oxygen Uptakes were measured by the conventional Warburg technique. Anaerobic experiments were carried out in Thunberg tubes (with shaking). After incubation one drop of concentrated hydrochloric acid was added to stop the reaction and the tubes placed in boiling water for 15 minutes to ensure sterility.

Unless otherwise stated incubations were carried out for the one hour at 38°C.

All results expressed below have been corrected for their appropriate blanks. All concentrations are expressed as final concentrations.

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[1]This work was supported in part by a Commonwealth Research Grant.

RESULTS.

The production of ammonia from aspartic acid by *Proteus* X-19 cells incubated aerobically, is markedly in excess of that occurring anaerobically (Table 1). Addition of 10^{-2}M cyanide, however, reduces this aerobic activity to the anaerobic value, which itself is insensitive to cyanide. Although the aerobic deamination is accompanied by an uptake of oxygen, which is also cyanide sensitive, it is not necessarily indicative of the presence of an aspartic acid oxidase; the results are amenable to an entirely different interpretation (see Discussion).

TABLE 1.

Effect of cyanide and anaerobiosis on deamination of aspartic acid.

L-aspartate, 0·0025 M; KCN, 0·01 M; Buffer, 0·06 M phosphate, pH 7·2; Cells, 0·36 mg. dry wt.; Final volume, 3 ml.

Conditions	QNH_3*	QO_2*
Aerobic, no KCN	316	266
Aerobic, + KCN	100	0
Anaerobic, no KCN	102	
Anaerobic, + KCN	104	

* $QNH_3 = \mu l\ NH_3$/mg. dry wt. cells/hr. $QO_2 = \mu l\ O_2$/mg. dry wt. cells/hr.

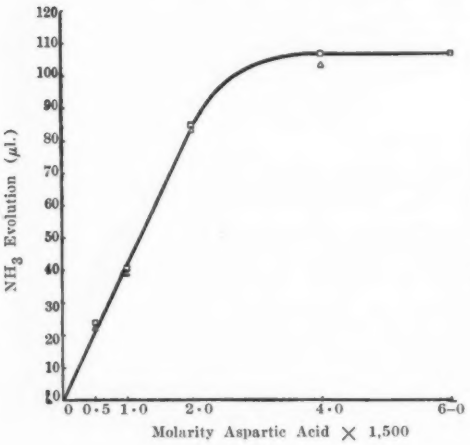

Fig. 1.

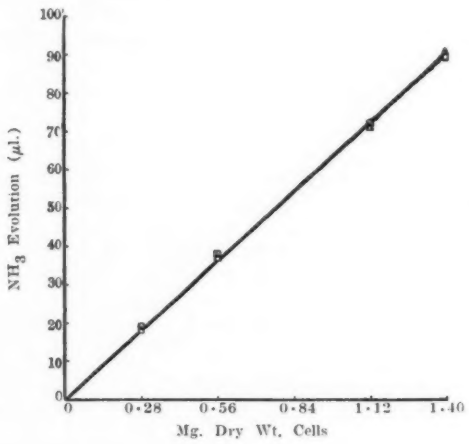

Fig. 2.

Fig. 1. Effect of Substrate Concentration on the Deamination of Aspartic Acid.
Conditions: Buffer, 0·06 M phosphate pH 7·0; Cells, 1·0 mg. dry wt.; Final volume, 3 ml.
Triangles: No additions — incubated anaerobically.
Squares: 10^{-2}M KCN added—incubated aerobically.

Fig. 2. Effect of Cell Concentration on the Deamination of Aspartic Acid.
Conditions: L-aspartate, 0·004 M; Buffer, 0·06 M phosphate pH 7·2; Final volume, 3 ml. Triangles and squares the same as for Fig. 1.

Further evidence that the ammonia liberated aerobically in the presence of cyanide is due to the "anaerobic" system, is given in Figs. 1 and 2 which show the effect of substrate concentration and cell concentration respectively.

The experiments reported in the remaining part of this paper were all carried out anaerobically.

Effect of pH.

Fig. 3 shows that the pH for optimum anaerobic deamination lies between pH 6·8 and pH 7·2.

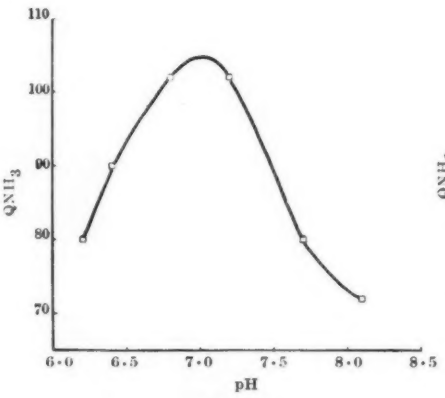

Fig. 3.

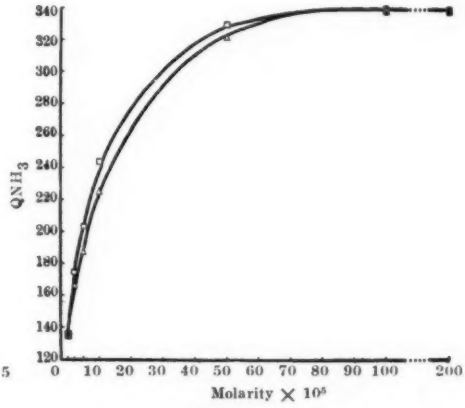

Fig. 4.

Fig. 3. Influence of pH on the Anaerobic Deamination of Aspartic Acid.

Conditions: L-aspartate, 0·004 M; Cells, 1·0 mg. dry wt.; Buffers, 0·06 M phosphate*; Final volume, 3 ml.

* pH values were determined with a Jones', Model B, spectrometer.

Fig. 4. Effect of Glucose and Adenosine Concentration on Anaerobic Deamination of Aspartic Acid.

Conditions: L-aspartate, 0·0025 M; Cells, 0·27 mg. dry wt.; Buffer, 0·06 M phosphate pH 7·2; Final volume, 3 ml. Squares: Glucose. Triangles: Adenosine.

Effect of carbohydrates.

Table 2 shows the effects of various carbohydrates and their derivatives on the anaerobic deamination system.

Those substances capable of being fermented by the organism stimulate the output of ammonia; glucose, lactate, glycerol and maltose were the most active. The literature on carbohydrate fermentation by *P. vulgaris* is scant, but from those results which have been published it would appear that the stimulation produced by the sugars is roughly correlated with their ease of fermentation. (Wenner and Rettger, 1919; Yacob, 1931-32; Rustigan and Stuart, 1945).

The action of formate is interesting in that Gale (1938), working with *E. coli*, found that while formate activated his "Aspartase II" aerobically, little or no such effect occurred anaerobically.

TABLE 2.

Effect of carbohydrates etc., on the anerobic deamination of aspartic acid.

L-aspartate, 0·0033 M; Additions 0·001 M; Buffer, 0·06 M phosphate pH 7·2; cells, (mg. dry wt.); Exp. 1, 0·32; Exp. 2, 0·28; Exp. 3, 0·19; Exp. 4, 0·26; final volume, 3 ml.

Expt.	Additions	QNH_3	Expt.	Additions	QNH_3
1	None	122	2	None	143
	Glucose	309		Glucose	341
	Fructose	155		Pyruvate	285
	Galactose	189		Oxalacetate	168
	Mannose	156		Lactate	353
	Lactose	144		Acetate	143
	Maltose	275		Formate	200
	Sucrose	194		Succinate	146
	Cellobiose	178		Malate	111
	Raffinose	206		Fumarate	146
3	None	100	4	None	73
	Glucose	268		Glucose	204
	Ribose	121		Cis-aconitate	69
	Xylose	121		Trans-aconitate	58
	Arabinose	116		Citrate	89
	Rhamnose	100		α-ketoglutarate	112
	Glycerol	258		Acetaldehyde	70
	Ethanol	105		Formaldehyde	69
				Acetone	73

Of interest also is the stimulation produced by α-ketoglutarate since, if stimulation is dependent upon fermentation, it would indicate that α-ketoglutarate can be metabolized by this organism.

The inhibitions by trans-aconitate and malate remain unexplained though in the latter case it may be due to a product of reaction effect in the system, aspartate $\rightleftharpoons$ malate, $\rightleftharpoons$ fumarate the inactivity of fumarate being due to permeability factors.

Effect of purine derivatives.

Gale (1938) has shown that adenosine and, to a lesser extent, adenylic acid and adenine, reactivated the "aspartase" of *E. coli* after it had been partially inactivated by standing at 0°C. for three hours. Adenylic acid and adenosine also stimulated slightly the initial activity of the cells. In the experiments of Lichstein and Christman (1948), adenylic acid reactivated the cells of a number of bacterial species whose deaminating activity toward aspartate had been diminished by incubation at pH 4·0. Adenine and guanine also had a slight effect.

From Table 3 it can be seen that adenylic acid and adenosine at a concentration of 10^{-3}M stimulate the anaerobic activity of *Proteus* X-19 to the same extent as glucose, while guanosine is only slightly less effective at this concentration. Adenine and guanine, however, appear to have no effect under these conditions.

The marked stimulations produced by adenosine and adenylic acid indicate that the system under consideration is analogous to the "Aspartase II" of *E. coli*. This is borne out further by the fact that treatment of the cells with toluene almost completely abolished deaminase activity (Table 5) while stimulation was inhibited 100 per cent by this treatment. (Gale 1938).

The influence of glucose and adenosine concentrations on deaminase activity is shown in Fig. 4. Both reach their optimal concentration at *ca.* 8×10^{-4}M.

TABLE 3.

Effect of purine derivatives on the deamination of aspartic acid.

Cells, 0·32 mg. dry wt. Other conditions same as for Table 2.

Additions	QNH_3
None	88
Glucose	236
Adenylic Acid	240
Adenosine	247
Guanosine	215
Adenine	91
Guanine	91

TABLE 4.

Effect of sulphydryl compounds on the deamination of aspartic acid.

L-aspartate, 0·0033 M; Buffer, 0·06 M phosphate pH 7·2; Cells, 0·29 mg. dry wt; Final volume 3 cc.

Additions	Concentration	QNH_3
None	—	110
Glucose	1×10^{-3}M	280
Cysteine	3×10^{-3}M	110
Glutathione	1×10^{-3}M	166
Glutathione	3×10^{-5}M	157

Effect of sulphydryl compounds.

Gale (1938) has shown that in *E. coli* the deamination of aspartic acid is stimulated by the addition of small amounts of cysteine, but not of glutathione, to the reaction medium. In *Proteus* X-19 the opposite situation exists, no stimulation being observed with cysteine in concentrations as high as 3×10^{-3}M. (Table 4).

In the case of serine deaminase, an enzyme which appears to be similar, in many respects, to "Aspartase II," (Gale and Stephenson, 1938; Lichstein and Christman, 1948) the cell-free system isolated by Wood and Gunsalus (1949) required both adenylic acid and glutathione for activity. However, Gale's cell-free preparation of "Aspartase II" apparently needed adenosine only for its action (Gale 1938).

Effect of biotin.

Lichstein *et al.* (Lichstein and Umbreit, 1947; Lichstein and Christman, 1948) have shown that cells of various bacterial species, rendered biotin deficient by incubation at 20-30°C. at pH4, possess diminished deaminase activity toward aspartate, threonine and serine. The activity could be regenerated however, by the addition of biotin or adenylic acid. (See also Wright, Cresson and Skeggs, 1940.) In the present study the action of biotin, alone and in combination with glucose, adenosine and glutathione, was followed using:

(a) fresh cells and (b) cells whose deaminating power had been reduced by incubation in distilled water at 38°C.

In no instance was any stimulatory action obtained with biotin concentrations up to 5×10^{-6}M, a concentration which produced a marked effect in the experiments of Lichstein and Christman.

Unfortunately the biotin available was insufficient for further work.

TABLE 5.

Effect of toluene treatment on deamination of aspartic acid.

L-aspartate 0·0025 M; Additions 0·001 M; buffer 0·06 M phosphate, pH 7·2; Final volume 3 ml.

Expt.	Additions	Toluene concentration(1) ml. per 10 ml. suspension	Untreated cells QNH_3	"Toluene" cells QNH_3
1	None	1·00	93	10
2	None	1·00	129	16
3	None	1·00	80	11
4	None	0·50	94	8
5a	None	0·25	113	20
5b	Glucose	0·25	220	18
5c	Adenosine	0·25	218	18
6a(2)	None	0·25	57	13
6b	Glucose	0·25	197	13
6c	Adenosine	0·25	201	14
7a(2)	None	0·20	56	15
7b	Glucose	0·20	192	12
7c	Adenosine	0·20	189	14

(1) The suspensions were shaken with toluene for 3-5 minutes at room temperature. The toluene was then allowed to separate and was removed before the suspensions were used.

(2) The organisms used in experiments 6 and 7 were grown in nutrient broth of pH 7·6 for 15 hours at 37°C.

Function of glucose and adenosine.

Both Gale (1938) and Lichstein and Christman (1948) have postulated that adenosine is utilized in the formation of some coenzyme-like factor, the latter workers suggesting biotin to be an integral part of this coenzyme. A logical extension to this theory would be that fermentable substances promote the production of the coenzyme by virtue of the energy obtained from their metabolism.

A number of experiments were performed to test this hypothesis.

The course of deamination.

If a coenzyme-like factor were being synthesized it might be possible to demonstrate a lag phase during the early stages of the stimulated reaction corresponding to a period during which the concentration of the cofactor was insufficient for optimal stimulation. Fig. 5 shows the effect of adenosine and glucose on the course of reaction. The unstimulated enzyme had a relatively low initial activity which suffered a gradual decline over the time interval of the experiment. With excess (10^{-3}M) glucose or adenosine, optimal activity was observed at the 10 minute interval, the reaction over the hourly period being linear. As would be expected from Table 3, it has been shown that the curves with adenosine or glucose coincide, that is both adenosine and glucose, at this concentration produced maximal stimulation.

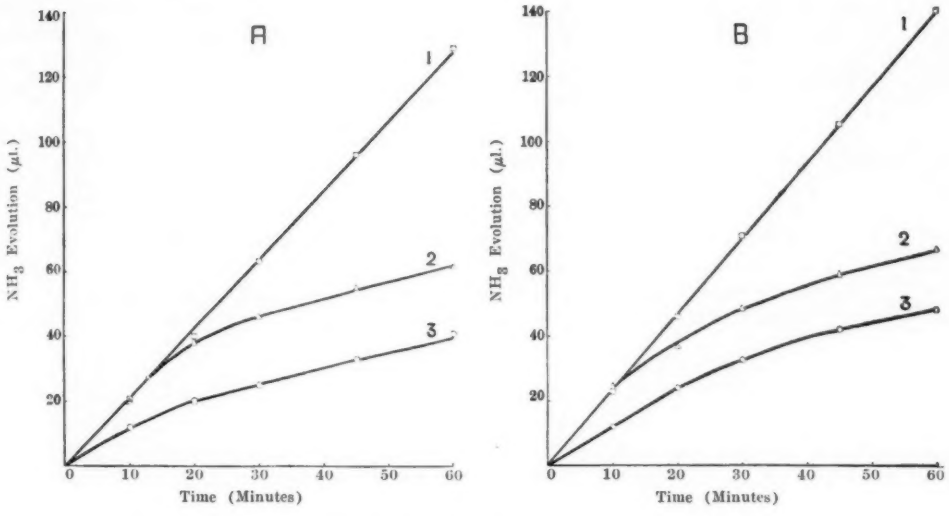

Fig. 5. The Course of Anaerobic Deamination.

Conditions: L-aspartate, 0·0025 M; Buffer, 0·06 M phosphate pH 7·2; Final volume, 3 ml.

Part A: Cells, 0·52 mg. dry wt. Curve 1: 10^{-3}M glucose added; curve 2: 5×10^{-5}M glucose added; curve 3: No additions.

Part B: Cells, 0·30 mg., dry wt. Curve 1: 10^{-3}M Adenosine added; curve 2: 5×10^{-5}M adenosine added; curve 3: No additions.

Addition of a sub-optimal concentration (5×10^{-5}M) of adenosine or glucose produced, during the first 10-15 minutes, stimulation identical with that produced by excess of these substances. Thereafter, the action diminished rapidly until the course of reaction followed that of the unstimulated system.

It will be noticed that after the first 20-30 minutes the reaction of the unstimulated controls tended to become linear. The initial enhanced rate of reaction could be due to stimulatory substances within the cells as prepared. Accordingly, attempts were made to nullify this effect by preincubating the cells at 38°C., for 30-45 minutes before addition of the substrate. In no instance, however, could a purely linear curve be obtained. Nevertheless the inital stimulation was, in all cases, markedly reduced indicating that some stimulatory substances were being reduced in concentration during this preincubation period. This would indicate also that the effectiveness of these stimulants is nullified more rapidly in the presence of deamination than by "straight metabolism." This is further exemplified by experiments with added glucose or adenosine. When added together with aspartate, the action of a 5×10^{-5}M concentration of these substances apparently ceased within 30 minutes (Fig. 5). However preincubation for 30 minutes before the addition of the substrate did not totally abolish their effect but merely reduced their activity (Table 6). It would be tempting to postulate that only the initial stimulated output of ammonia is a manifestation of "Aspartase II" activity the latter, linear, production being due to "Aspartase I". The criterion for this latter enzyme is insensitivity to toluene. (Gale, 1938; Quastel and Woolf, 1926). Table 4 shows that even mild treatment such

as shaking with toluene at room temperature for 3-5 minutes resulted in a reduction of deamination activity to a QNH_3 of from 8-20. On the other hand extrapolation of the linear portion of the time curves of non-treated cells (e.g. Fig. 5) always gave values equivalent to a QNH_3 of between 50 and 80.

There are three ways in which these results could be explained:

(a) Perhaps Gale's assumption that Aspartase I is insensitive to toluene is not true. Admittedly his cell free preparation of this enzyme (Gale 1938) proved so, but the situation in the living cell may be different. Moreover there may be a species difference between *E. coli* and *Proteus* X-192.

(b) The "Aspartase II" system may be able to work in a linear fashion without the presence of stimulatory substances, or

(c) A third enzyme may be present, sensitive to toluene but unaffected by glucose, adenosine etc. At the moment it is impossible to differentiate between these three possibilities.

Effect of preincubation.

The results in Fig. 5 are not suggestive of a coenzyme synthesis but rather of a direct action of adenosine and glucose in the deamination reaction. The results obtained by preincubating the cells with glucose or adenosine prior to the addition of aspartate, also do not hold with the coenzyme theory (Table 6).

The experiments were carried out as follows. Two sets of Thunberg tubes were arranged. In one set the adenosine or glucose was placed in the side bulb (SB) with aspartate as usual. In the other set the additions were placed in the tube (T) with the cells. The tubes were then evacuated as rapidly as possible and incubated for 30 minutes before tipping in the contents of the side bulb.

If glucose and adenosine were concerned in the synthesis of a coenzyme one would expect, when they were preincubated with the cells, if not greater stimulation, at least an effect equal to that obtained when the stimulants were added directly. In no case was any significant enhancement of stimulation observed. When glucose or adenosine was in excess (10^{-3}M) the stimulation after preincubation was the same as when these substances were added with aspartate. At suboptimal concentrations, however, preincubation markedly decreased their stimulatory actions, suggesting once again a more direct relationship to the enzyme system which is impaired by preincubation, presumably by partial removal of the stimulants by other metabolic processes.

The effect of preincubation was tested further in a series of short term experiments in which glucose or adenosine was incubated for periods of 5, 10, 20 and 30 minutes before the addition of substrate and the liberated ammonia estimated in the first 15 minutes after tipping. In no instance was there any significant difference in these results from those in which the stimulants were added together with the substrate.

Influence of cell concentration.

If a cofactor were being synthesized one might expect increased stimulation with increasing cell concentration, since the amount of coenzyme synthesized should be greater and, if diffusible (Gale 1938), would result in a larger "coenzyme pool" in the reacting medium.

The converse occurred under the experimental conditions employed (Table 7). Increased activity per unit cell mass was obtained with decreasing cell concentration.

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[2]Since this paper was written it has been shown by the author that toluene treatment produces a considerable inactivation of Aspartase I also, the original activity being regenerated upon addition of some metallic ions such as Mg.++, Mn++ and Co++.

TABLE 6.

Effect of pre-incubations with adenosine or glucose on aspartic acid deamination.

L-aspartate, 0·0025 M; Cells (mg. dry wt.) Ex. 1, 0·50; Ex. 2, 0·45; Buffer, 0·06 M phosphate pH 7·2; Final volume 3 ml.

Incubation: 30 minutes before and after tipping.

Expt.	Addition	Concentration	Position	μl. NH_3 in 30 mins.
1	None			32
	Glucose	2×10^{-3}M	S B	78
	Glucose	2×10^{-3}M	T	79
	Glucose	1×10^{-4}M	S B	61
	Glucose	1×10^{-4}M	T	45
	Glucose	5×10^{-5}M	S B	52
	Glucose	5×10^{-5}M	T	40
2	None			23
	Adenosine	2×10^{-3}M	S B	85
	Adenosine	2×10^{-3}M	T	90
	Adenosine	1×10^{-4}M	S B	58
	Adenosine	1×10^{-4}M	T	37
	Adenosine	5×10^{-5}M	S B	46
	Adenosine	5×10^{-5}M	T	28

S B = side bulb. T = Tube.

TABLE 7.

Influence of cell concentration on the stimulation of aspartic deaminase.

L-aspartate, 0·0025 M; Glucose and Adenosine 5×10^{-5}M; Buffer 0·06 M, phosphate pH 7·2. Final volume, 3 ml.

Expt.	Mg. dry wt. cells per 3 ml.	No addtns. QNH_3	+ Glucose QNH_3	+ Adenosine QNH_3
1	0·24	121	210	196
	0·18	125	233	203
	0·12	129	262	238
2	0·26	123	190	169
	0·195	128	213	190
	0·13	120	246	223

Thus stimulation by suboptimal concentrations of glucose or adenosine is inversely related to cell concentration, indicating that these substances are utilized directly and once metabolized are unable to affect the reaction. That there is no direct proportionality between cell concentration and stimulation is not a hindrance to this view since the amounts of stimulatory substances present within the cell and the extra metabolism of glucose and adenosine are unknown factors which will markedly affect the $\frac{\text{stimulation}}{\text{cell concentration}}$ ratio.

Stimulation by excess concentrations of glucose and adenosine was independent of cell concentration as would be expected from the foregoing results.

TABLE 8.

Combined effect of glucose and adenosine on aspartic acid deaminase.

L-aspartate, 0·0025 M; Buffer, 0·06 M phosphate, pH 7·2; Cells (mg. dry wt.), Ex. 1, 0·58; Ex. 2, 0·31; Ex. 3, 0·31; Ex. 4, 0·25; Ex. 5, 0·40.

	Additions	Concentration	QNH_3				
			Ex. 1	Ex. 2	Ex. 3	Ex. 4	Ex. 5
A	None		93	129	123	144	
	Glucose	5×10^{-5}M	123	190		224	
	Adenosine	5×10^{-5}M	125	190		228	
	Glucose	1×10^{-4}M	145	226		264	217
	Adenosine	1×10^{-4}M	144	224		258	216
	Glucose + Adenosine	5×10^{-5}M + 5×10^{-5}M	144	210		236	190
B	Glucose	1×10^{-3}M			302	356	
	Adenosine	1×10^{-3}M			300	356	
	Glucose	2×10^{-3}M			302	352	315
	Adenosine	2×10^{-3}M			297	356	319
	Glucose + Adenosine	1×10^{-3}M + 1×10^{-3}M			277	324	290

TABLE 9.

Glutathione on adenosine and glucose stimulation.

L-aspartate 0·0025 M; Buffer 0·06 M phosphate pH 7·2; Cells (mg. dry wt.), Ex. 1, 0·2, Ex. 2, 0·4; Ex. 3, 0·24; Ex. 4, 0·47; Final volume 3 ml.

Additions	Concentration	QNH_3			
		Ex. 1	Ex. 2	Ex. 3	Ex. 4
None		110	127	156	132
GSH	1×10^{-3}M	165	172	200	164
Glucose	5×10^{-5}M	230	191	224	181
Adenosine	5×10^{-5}M	200	186	216	179
Glucose + GSH	5×10^{-5}M + 1×10^{-3}M	245	208	252	198
Adenosine + GSH	5×10^{-5}M + 1×10^{-3}M	230	207	242	
Glucose	1×10^{-3}M		307	316	
Adenosine	1×10^{-3}M		305	312	
Glucose + GSH	1×10^{-3}M + 1×10^{-3}M		302	316	
Adenosine + GSH	1×10^{-3}M + 1×10^{-3}M		310	322	

Influence of glucose in combination with adenosine.

When glucose and adenosine are added together in suboptimal amounts to the deaminase system, the stimulation produced is not additive, nor does one enhance the effect of the other (Table 8, Section A). Thus a combined concentration of 10^{-4}M resulted in a QNH_3 of the order of that produced by either 10^{-4}M adenosine or glucose alone. Actually the combined QNH_3 was very often slightly lower than that produced by either substance *per se*, a result which was also obtained with higher concentrations (Table 8, Section B). However, this effect was never more than about 10 p.c. of the total activity and so it is unlikely that it represents any significant antagonism between adenosine and glucose.

Effect of glutathione on adenosine and glucose stimulation.

From Table 9 the following facts can be seen. Glutathione had no effect on the production of ammonia in the presence of high concentrations of adenosine or glucose. With low concentrations of these substances glutathione did produce a slight increase in QNH_3 over that produced by adenosine or glucose alone. This increase, however, was very small compared with that produced by glutathione alone.

DISCUSSION.

It is obvious from the foregoing account that cells of *Proteus* X-19, harvested under the experimental conditions used, have, at the most, only 30–40 p.c. of their potential ability to deaminate aspartic acid anaerobically. Addition of some fermentable substances such as glucose, lactate, etc., or adenosine and related compounds can produce a marked increase in the deaminating activity of the cells. Glutathione, on the other hand, although producing appreciable stimulation at very low concentrations, never attains the efficiency of adenosine or glucose in this respect. Table 9 would indicate that adenosine and glucose can duplicate the action of glutathione since the effect of the latter substance is nullified by the presence of either of the other compounds. If we assume the action of glutathione to be dependent upon its reducing power (cf. Gale, 1938, who postulated that a reducing substance is necessary for "Aspartase II" activity in *E. coli*) then the functions of glucose and adenosine must also be partially due to their reducing capacities. The slight stimulation brought about by formic acid could also be explained on this basis.

The marked superiority of adenosine and glucose etc., indicates that they must also have some other rôle. A significant fact is that, in all the experiments performed, the results obtained with adenosine were almost identical with those with glucose. It would seem, therefore, either that their actions are identical or that, though the actual mechanisms may differ, they act at a common point in the deaminase system. The theory that adenosine may act by virtue of energy liberated during its metabolism appears invalid since in no case was any significant ammonia produced from adenosine *per se*, while in *E. coli* and other organisms ammonia is a characteristic end product of adenosine metabolism (Lutwak-Mann, 1936). Moreover, its immediate breakdown

products, adenine and ribose, do not themselves stimulate to any significant degree.

An alternative explanation, particularly in the case of glucose and its breakdown products, could be that these substances act anaerobically as hydrogen acceptors in an oxidative type of deamination. This theory is difficult to reconcile, however, with the superiority of lactate over oxalacetate and pyruvate.

The results listed in Tables 6 and 7 and Fig. 6 are not in favour of the theory that adenosine and glucose contribute to the synthesis of a coenzyme but support the view that they participate directly in the deamination system, acting apparently in a non-catalytic fashion since the activity of sub-optimal amounts decreases rapidly after the initial stimulation. Further conjecture would, however, be pointless until more details of the reaction are known.

The inactivity of biotin was at first surprising in view of Lichstein and Christman's (1948) results. Axelrod *et al.* (1948) were also unable to demonstrate biotin stimulation of aspartic acid deamination under similar conditions to those of Lichstein and Christman, whereas adenosine produced almost 100 p.c. reactivation of the aged cells. Lichstein (1949), however, has demonstrated that this discrepancy is due to a difference of medium upon which the cells were grown. This is probably also the explanation for the negative results reported here, since the growth medium was similar to that used by Axelrod. It may be significant that cell-free serine deaminase requires only glutathione and adenylic acid for activity (Wood and Gunsalus, 1949), whereas both biotin and adenylic acid acted on this enzyme in the resting bacterial cell in a similar way to that on the aspartic deaminase system (Lichstein and Christman, 1948). It is of course difficult to draw comparisons because of the different conditions used. It is not stated whether aerobic or anaerobic conditions were used in Lichstein and Christman's experiments. If the former then further metabolism of the fumarate formed during the deamination reaction would in all probability have stimulated the initial process. These same workers described fumarate metabolism in their organism as proceeding through malate and oxalacetate to pyruvate (Lichstein and Umbreit, 1947b), and obtained evidence for a stimulation of the oxalacetate→pyruvate reaction by their biotin preparation.

Remembering the superiority of pyruvate over oxalacetate in enhancing aspartic acid deaminase activity (Table 2), this fact could explain also the stimulation of this system by biotin observed by Lichstein and his co-workers.

The same reasoning could be used to explain the superior ammonia output observed aerobically with *Proteus* X-19 (Table 1). That is instead of being due to an oxidative deaminase, the excess ammonia could result from stimulation of the "anaerobic" system by the oxidative metabolism of fumarate. This latter hypothesis finds support in the fact that the rate of fumarate

oxidation by *Proteus* X-19 is virtually identical over an hourly period with that of aspartate. Moreover, particularly with old cells (36 hours in buffer) in which activity has decreased markedly, a lag period of 10–20 minutes may be observed with aspartate (but not with fumarate), the rate thereafter increasing rapidly to parallel that with fumarate (unpublished results).

Thus oxidation of fumarate could account for the oxygen consumption observed with aspartate, the lag period with the latter observed with deteriorated cells being due to insufficient fumarate for significant oxidation.

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SUMMARY.

The anaerobic deamination of aspartic acid by *Proteus* X-19 is stimulated by fermentable carbohydrates and some derivatives, certain purine derivatives and by glutathione.

Biotin did not increase the activity of fresh or "aged" cells alone or in combination with glucose, adenosine, or glutathione.

The stimulations produced by adenosine and by glucose appear to be identical and reach an optimum at *ca.* 8×10^{-4}M under the conditions used.

The action of glutathione is duplicated by either glucose or adenosine.

The significance of these results in the elucidation of the mechanism of stimulation is discussed.

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THE INFLUENCE OF CLOVER LEYS ON THE THIAMIN AND NITROGEN CONTENTS OF WHEAT[1]

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In an earlier paper, Lee, Clare and Underwood (1949) have presented the results of a study of the influence of comparatively short rotations (maximum four years) on the thiamin, nitrogen and riboflavin contents of wheat grown in Western Australia and South Australia. Small increases in the average thiamin and nitrogen contents of the wheat from the rotations which included one or two years of crop legume were found, but no obvious relationship between riboflavin content and rotation was discernible.

In view of these findings it was thought desirable to investigate the effects of much longer rotations, including a number of years of clover pasture, on the nitrogen and thiamin contents of wheat. The results of such an investigation are presented in this paper. An indication that such long rotations might have a marked influence was provided by an examination of composite samples of wheat (variety Baldmin) grown on "clover" land and "non-clover" land at Rutherglen Research Station, Victoria, in 1948. The wheat from the land which had been under subterranean clover pasture for 10 years contained an average of 3·6 μg./gm. of thiamin, whereas the wheat from the wheat-oats-fallow and fallow-wheat rotations contained an average of 2·7 μg./gm. of thiamin.

MATERIALS AND METHODS.

The wheat samples were obtained for the 1949 season from an experiment conducted by the W.A. Department of Agriculture at Narrogin and from the Soil Regeneration Trial and the Soil Fertility Trial conducted by the Victorian Department of Agriculture at Rutherglen Research Station and at Dookie Agricultural College respectively.

The samples from Rutherglen consisted of three replications of the variety Baldmin from each of the following treatments:

(i) Fallow-wheat rotation, first crop after eleven years of subterranean clover ley.
(ii) Continuous wheat rotation, first crop after eleven years of subterranean clover ley.
(iii) Fallow-wheat-oats rotation, *never under ley.*

The third treatment was from the same soil type as treatments (i) and (ii) and adjacent to them but was not part of the same experiment. A statistical analysis of the significance of differences between treatments (i) and (ii) and treatment (iii) was therefore not possible.

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The samples from Dookie were of the variety Quadrat sown in 1949 on an area which had received different treatments since 1940. There were two replications from each of the following rotations:

(i) Wheat, oats, fallow, since 1940, wheat 1949.
(ii) Clover pasture 1940–45, wheat 1946, oats 1947, fallow 1948, wheat 1949.
(iii) Clover pasture 1940–48, winter fallowed for wheat 1949.
(iv) Clover pasture 1940–48, autumn ploughed for wheat 1949.

The samples from Narrogin were from an experiment with the double objective of determining the suitability of certain varieties for this district and of comparing the yields and flour strength of these varieties when grown on old clover land and on land which had been under a typical three-course rotation (volunteer pasture, fallow, wheat). The "clover land" had been under subterranean clover pasture for 10 years prior to the autumn sowing of the 1949 wheat crop. The "non-clover land" had been under the three-course rotation for about 20 years prior to fallowing in 1948 for the 1949 wheat crop. There were four replications of each of the varieties Bencubbin, Yalta, Eureka and Kondut.

Methods of analysis.

Thiamin. The method used was essentially that of Slater (1946), who adapted the thiochrome method of Wang and Harris (1939) for use on cereals and cereal products. Our method differed slightly from the method of Slater in that the method of addition of reagents was that described by Hinton (1943). Readings were made with a "Spekker" fluorimeter using a "Woods Glass" ultra violet filter between the lamp and the fluorescent solution, and a Wratten 39 and a Wratten 2A filter between the solution and the photocell.

Nitrogen. The normal Kjeldahl method was used with selenium as catalyst. The ammonia was distilled into 4 p.c. boric acid, and directly titrated with standardized hydrochloric acid using methyl red-methylene blue mixed indicator.

RESULTS.

The individual results for each treatment at each location are given in an Appendix. As pointed out earlier, the data are not amenable to statistical analysis for significance of differences between the legume and non-legume rotations. However, the individual differences within treatments are in most cases very small, as will be seen from inspection of the Appendix. The results are therefore presented for convenience of discussion in a summarized form in Tables 1, 2 and 3 which give the mean values for thiamin and nitrogen for each treatment at Rutherglen, Dookie and Narrogin respectively.

TABLE 1.

Average N and thiamin contents of wheat at Rutherglen, Victoria. Variety Baldmin, 1949 season, results expressed on dry basis.

Treatment	Nitrogen* p.c.	Thiamin µg./gm.
(i) Fallow-wheat, first crop after 11 years, clover pasture	1·96	3·6
(ii) Continuous wheat, first crop after 11 years clover pasture	1·80	3·6
(iii) Fallow, wheat, oats rotation, never under clover pasture	1·40	2·7

* Calculated from figures for protein content kindly supplied by Mr. F. Penman, formerly of the Victorian Department of Agriculture.

Each of the figures in Table 1 is the mean of three replications. They show that the long period under clover pasture has resulted in marked improvement, of the order of 30 p.c., in both the nitrogen and thiamin contents of the wheat, compared with the "standard" three course rotation.

TABLE 2.

Average N and thiamin contents of wheat at Dookie, Victoria.
Variety Quadrat, 1949 season, results expressed on dry basis.

Treatment	Nitrogen* p.c.	Thiamin μg./gm.
(i) Fallow, wheat, oats rotation since 1940, wheat 1949	1·76	3·5
(ii) Clover pasture 1940–45, wheat 1946, oats 1947, fallow 1948, wheat 1949	1·89	3·6
(iii) Clover pasture 1940–48, winter fallowed for wheat 1949	2·20	4·5
(iv) Clover pasture 1940–48, autumn ploughed for wheat 1949	2·02	4·0

* Calculated from figures for protein content kindly supplied by Mr. F. Penman, formerly of the Victorian Department of Agriculture.

Each of the figures in Table 2 is the mean of two replications. They indicate a marked influence of the period of eight or nine years under clover ley in raising the nitrogen and thiamin contents of the wheat. This is particularly marked in the case of the third treatment following fallow, where the order of improvement over the levels in the wheat from the three-course rotation (treatment (i)) is similar to that obtained in the Rutherglen experiments, namely 25–30 p.c. It is noteworthy that in treatment (ii) where the 1949 wheat crop was preceded by two cereal crops and a fallow year following six years of clover ley there has been no such effect on the thiamin content of the wheat and only a very slight effect on the nitrogen content.

TABLE 3.

Average N and thiamin contents of wheat at Narrogin, W.A.
1949 season, results expressed on dry basis.

	Bencubbin		Yalta		Eureka		Kondut	
Treatment	N p.c.	Thiamin μg./gm.	N p.c.	Thiamin μg./gm.	N p.c.	Thiamin μg./gm.	N p.c.	Thiamin μg./gm.
Clover pasture 1938–48 autumn ploughed for wheat 1949	1·77	3·8	2·11	4·7	1·90	4·5	2·09	4·8
Fallow-wheat-volunteer pasture rotation since 1930—never under clover pasture	1·45	3·3	1·76	3·9	1·61	3·5	1·61	3·7

Each of the figures in Table 3 is the mean of four replications. All four varieties are appreciably higher in nitrogen and thiamin when grown after a clover ley than when grown on fallow following a number of years cropping. The effect is less pronounced, however, particularly in the case of thiamin, in the variety Bencubbin than in the other three varieties.

Statistical examination of these results for differences between varieties revealed the fact that the variety Bencubbin is significantly lower in both nitrogen and thiamin than the other

three varieties. This confirms the earlier finding of Lee and Underwood (1950), from a study of wheat variety trials in Western Australia, that there is a significant effect of variety on both the thiamin and nitrogen contents of wheat grain.

Nitrogen-Thiamin correlation.

All the figures for nitrogen and thiamin from the different varieties at the three locations, as presented in the Appendix, were examined to determine the correlation between nitrogen and thiamin contents. For the degrees of freedom present a highly significant positive correlation ($r = +0{\cdot}645$) was found. This is very similar to the correlations obtained ($r = +0{\cdot}702$ and $r = +0{\cdot}614$) from the two previous studies of Lee *et al.* (1949) and Lee and Underwood (1950), where several varieties were involved. It is, however, very much lower than the correlation found by the latter workers for the individual varieties Bungulla ($r = +0{\cdot}845$) and Charter ($r = +0{\cdot}814$).

DISCUSSION.

In recent years a number of workers in southern Australia (Shier and Cullinane, 1948; Woodroffe, 1949; Bath, 1949; Sims and Jardine, 1949) have presented the results of experiments demonstrating the value of the inclusion of two or more years of a crop or pasture legume in the rotation in increasing the yields of wheat. These workers have also presented some evidence indicating that these increases in yield are accompanied by improvements in the nitrogen content and, in the case of Shier and Cullinane (1948), upon the "flour strength" of the wheat. Lee, Clare and Underwood (1949) confirmed these findings in relation to nitrogen content and showed further that the inclusion of two years of a crop legume in the rotation resulted in similar small improvements in the thiamin content of the grain.

The importance of the present findings lies in the fact that, under a system of ley farming in which the wheat crop is preceded by a number of years of clover pasture, the improvement in quality of the wheat grain is much more marked than in the shorter rotations. This improvement seems to apply equally to nitrogen and thiamin and is largely, although not entirely, independent of variety. The increases resulting from the clover ley at different locations and with different varieties ranged from 16 to 30 p.c. but the overall improvement averaged 21 p.c. in the case of nitrogen content and 24 p.c. in the case of thiamin content. The actual mean figures for all samples of wheat grown on clover ley land were 1·99 p.c. nitrogen (11·7 p.c. protein) and 4·2 μg./gm. of thiamin, compared with 1·64 p.c. (9·6 p.c. protein) and 3·4 μg./gm. for the wheats grown on non-clover land. Increases of this order are of considerable significance in relation to the nutritive value of the grain and the baking quality of the flour.

Food consumption surveys carried out in 1946–7 showed that in the average Australian household approximately one-third of the total thiamin comes from bread and cereals. These surveys showed further that many households consumed less than the "recommended" allowances for thiamin. Various means of raising the thiamin intake have been put forward, of which the use of higher extraction flour, as in Great Britain, and the addition of

synthetic thiamin, as in U.S.A., have received the most publicity. It is not proposed to enter, in this paper, into a discussion of the merits of these or other proposals. They have been discussed recently by Hipsley (1950) and Underwood (1950). It is clear, however, that an effective means of appreciably raising thiamin intake lies in the adoption of ley farming methods, so that an increased proportion of the Australian wheat crop is grown on clover ley land. Pasture legumes already exist, which are suitable for this purpose over extensive wheat-growing areas in southern Australia. But the development and establishment of more productive types, adapted particularly to the drier parts of the wheat belt, constitute major problems for the plant breeder and agrostologist.

In some respects the improvement in the nitrogen content of wheat which results from the adoption of ley farming methods is even more important. Great prominence has been given recently to the low flour strength of much of the wheat now grown in Australia, particularly in Western Australia and Victoria where the experiments reported here were carried out. "Strength" of flour is now accepted as being dependent upon the quantity and quality of the gluten present. However, within varieties, "strength," for all practical purposes, is a function of the quantity of gluten, that is, of the nitrogen content of the grain. In view of the fact that substantial improvements in nitrogen content were shown by all varieties included in the present study, when these varieties were grown on clover ley land, it is clear that a means exists of raising substantially the baking quality of much of the Australian wheat crop without any change in the varieties grown. This does not mean, of course, that better varieties are not needed or that the problem of combining high yielding ability and high baking quality in new varieties is not still a major task for the cereal breeder.

SUMMARY.

The thiamin and nitrogen contents of wheat samples from experiments in Western Australia and Victoria were determined for the 1949 season.

Wheat grown on land which had been under subterranean clover ley for 8–11 years was found, at each location and with each variety examined, to be markedly higher in thiamin and nitrogen than wheat grown on adjacent "non-clover" land which had been frequently cropped during the preceding five or more years.

The average improvement for all samples was found to be 21 p.c. (range 15–40 p.c.) in the case of nitrogen content and 24 p.c. (range 15–33 p.c.) in the case of thiamin content.

The actual mean figures for wheat grown on clover ley land were 1·99 p.c. nitrogen (11·7 p.c. protein) and 4·2 μg./gm. of thiamin and for wheat grown on non-clover land 1·64 p.c. nitrogen (9·6 p.c. protein) and 3·4 μg./gm. of thiamin.

A highly significant positive correlation ($r = +0{\cdot}645$) was found between the nitrogen and thiamin contents of the samples.

The importance of these findings in relation to the nutritive value and baking quality of wheat is discussed.

Acknowledgments. It is a pleasure to record our indebtedness to Mr. F. Penman, formerly of the Victorian Department of Agriculture and to Mr. H. G. Cariss, Agricultural Adviser of the Western Australian Department of Agriculture, for their co-operation in supplying wheat samples.

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APPENDIX.

INDIVIDUAL RESULTS OF REPLICATIONS.

Narrogin, Western Australia.

Variety	Rotation	Nitrogen p.c.				Thiamin μg./gm.			
Bencubbin	Old clover land	1·73	1·76	1·80	1·81	3·7	3·6	3·9	3·9
	Fallow—no clover	1·41	1·41	1·46	1·54	3·3	3·3	3·3	3·5
Yalta	Old clover land	2·17	2·19	2·08	2·00	4·8	4·6	4·9	4·7
	Fallow—no clover	1·55	1·60	1·74	1·85	3·7	3·6	4·1	4·2
Eureka	Old clover land	1·83	1·88	1·97	1·94	4·1	4·4	4·9	4·5
	Fallow—no clover	1·67	1·52	1·60	1·65	3·6	3·3	3·6	3·6
Kondut	Old clover land	1·92	2·06	2·19	2·19	4·2	4·6	5·1	5·2
	Fallow—no clover	1·58	1·56	1·60	1·70	3·5	3·5	3·9	4·1

Rutherglen Research Station, Victoria

Rotation	Nitrogen p.c.			Thiamin μg./gm.		
1. Fallow-wheat, first crop after ley	1·89	1·97	2·02	3·5	3·5	3·8
2. Wheat continuous, first crop after ley	1·75	1·80	1·84	3·5	3·4	3·8
3. Fallow on oats control, never under ley	1·39	1·39	1·41	2·5	2·6	3·0

Dookie Agricultural College, Victoria.

Rotation	Nitrogen p.c.		Thiamin μg./gm.	
Pasture, 1940–45, wheat 1946, oats 1947, fallow 1948, wheat 1949	1·92	1·87	3·6	3·5
No pasture-wheat, oats fallow rotation, wheat 1949	1·82	1·70	3·4	3·6
Pasture 1940 till winter, fallowed 1948 for wheat 1949	2·15	2·25	4·6	4·4
Pasture 1940 till autumn, ploughed for wheat 1949	2·06	1·99	3·9	4·0

STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

VII. AN IMPROVED COMPLEMENT-FIXATION TECHNIQUE

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The earliest work on complement-fixation in influenza pointed to the existence of two distinct antigens in infected tissues (Wilson Smith, 1936). Subsequent investigations have confirmed this (Hoyle and Fairbrother, 1937a; Lennette and Horsfall, 1940; Friedewald, 1943; Henle, Henle, Groupé and Chambers, 1944), and have shown one antigen to be identical with or closely related to the virus particle, and the other to be a "soluble" substance, specifically concerned in the complement-fixing reaction.

In the important contribution of Wiener, Henle and Henle (1946), more detailed findings of a number of workers are summed up regarding the separation, size, etc. of the two antigens. In gravitational fields where the virus particle (600 S component) is brought down, the "30 S component" remains in the supernatant. This second antigen is sedimentable at 90,000 *g.* and has been found to have a diameter of about 10 mμ, which although small compared with the virus particle (approximately 100 mμ), is still quite large as colloids go. To avoid equivocal terms such as "small" or "soluble," the antigen detectable only in the complement-fixation test will be referred to as the CF-antigen, and its specific antibody as CF-antibody.

At the beginning of our studies on influenza immunology in mice, the old complement-fixation techniques not only seemed cumbersome but also fell short of our requirements in regard to accuracy and specificity. In fact it was only after a controlled experimental study of the separate factors involved in the complement-fixation test that a satisfactory technique could be evolved. This paper presents the results of these investigations and outlines the development of an improved technique aiming at maximal specificity, sensitivity, reproducibility and simplicity.

MATERIALS.

Ca-Mg-saline was prepared according to Mayer, Osler, Bier and Heidelberger (1946), as follows:

NaCl, 9·00 gm.; 5,5-diethyl barbituric acid, 0·575 gm.; Na-5,5 diethyl barbiturate, 0·375 gm.; $MgCl_2.6H_2O$, 0·168 gm.; $CaCl_2$, 0·028 gm.; Aq.dest., ad 1,000·00 gm.

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

The autoclaved solution had a pH of 7·25. This was used as standard diluent throughout.

Complement. Fresh serum of male guinea pigs.

Haemolytic system. 3 p.c. freshly washed sheep red blood cells sensitized with 8 HD_{50} of rabbit anti-sheep haemolysin.

Virus strains. The classical influenza strains, MEL (type A) and LEE (type B) were cultivated in the allantoic cavity of 11 day old hen eggs (Beveridge and Burnet, 1946) and the allantoic fluids harvested after 42 hours. Fresh fluids of high titre were used for animal inoculations.

Antisera were obtained from ferrets and mice convalescent from experimental infection with MEL (A) or LEE (B) virus.

CF-Antigen. 6–12 weeks old mice, in the acute stage of infection with MEL (A) or LEE (B) influenza viruses were killed under ether-chloroform (2:1) anaesthesia and their lungs harvested. The whole lung lobes were dissected out, and each wet mouse lung was ground in a mortar with sterile alundum, and suspended in 1·0 ml. Ca-Mg-saline. Each suspension was absorbed with human red blood cells to remove the virus or haemagglutinin. The resulting supernate will be referred to as the CF-antigen. For full details see below.

EXPERIMENTAL.

CF-Antigen.

Removal of virus.

Infected mouse lung suspensions contain appreciable amounts of virus as measured by the haemagglutination reaction (Fazekas de St.Groth and Donnelley, 1950a). This was removed by absorption with human red blood cells. Individual mouse lung suspensions were treated with 10 p.c. freshly washed human red blood cells for 30 minutes at 4° C., and then lightly centrifuged. Each supernate was checked for non-agglutination of 1 p.c. fowl cells in a 1:5 dilution.

Elimination of the anticomplementary property.

In the preliminary experiments all mouse lung suspensions were tested in the complement titration in dilutions of 1:1, 1:2·5, 1:5 and 1:10. Undiluted mouse lung always markedly reduced the titre of complement, while 1:2·5 or higher dilutions were not anticomplementary. Thus all mouse lung samples could be used with safety in a starting dilution of 1:5, i.e. at twice the non-anticomplementary level.

However, this anticomplementary property was again encountered in studies in the production of CF-antigen in experimentally infected mice (Fazekas de St.Groth and Donnelley, 1950a). Lungs harvested in the early stages of infection showed no fixation of complement when tested in a 1:5 dilution in the absence of standard antiserum, i.e. in saline controls. From the fourth or fifth day after infection, partial or complete fixation of complement occurred in the saline controls, and to compensate for this an increased amount of complement had to be used in the titration of these samples—an unsatisfactory procedure, as it decreased the sensitivity of the test. The significance of the appearance of this anticomplementary property was investigated in the following experiment.

A large number of 6 weeks old mice was inoculated intranasally with MEL (A) virus diluted to 10^{-4}, 10^{-5} and 10^{-6} in 10 p.c. horse serum saline. Five mice from each group were killed daily between the 2nd and the 11th day. Mice which died during this period were not included. The high death rate resulting from the 10^{-4} inoculum allowed this group to be followed only until the 8th day. Individual blood samples were taken for the estimation of serum complement-fixing antibody, and each mouse lung suspension was tested for CF-antigen by the standard technique (see below), and for its anticomplementary power in the following way.

A 1:5 dilution of each lung suspension was mixed with an equal volume of a large excess of complement (9 HD_{50}) and incubated for 60 minutes at 37° C. The "free" complement

was then tested, by diluting out the mixture, and adding to a standard volume (0·08 ml.) of each dilution an equal volume of sheep cells sensitized with 8 HD_{50} of haemolysin. The 50 p.c. lysis endpoint was read after a further 30 minutes at 37° C. Normal mouse lung suspensions were included as controls, and the anticomplementary property of the test samples is expressed in terms of the percentage of complement fixed, taking the normal lung level as zero. In Fig. 1 the daily CF-antigen and antibody titres and the percentage anticomplementary power for the 3 inocula are plotted.

The production of CF-antigen is similar for all inocula, reaching a maximum about the 4th day, and declining to the non-specific level on the 7th or 8th day. Likewise in all groups, antibody is first detected in the serum on the 7th or 8th day, and approaches its maximal level by the 10th or 11th day. In the period of antigen decline the anticomplementary property of mouse lung becomes apparent, rising sharply and starting to fall off again as the antibody appears in the serum. This would suggest an *in vivo* neutralization of antigen by antibody, with a peak of anticomplementary property, when the two are present in optimal proportions; when either is in excess, the phenomenon is not demonstrated. The effect is best shown in the 10^{-5} group where a marked fixation of complement is encountered on the 5th day, remaining at approximately the same level for several days, and decreasing by the 11th day. The high death rate resulting from the 10^{-4} inoculum made this a less satisfactory group to work with, but it does seem that the anticomplementary property appears early—the 4th day (c.f. 10^{-5}—the 5th day, and 10^{-6}—the 7th day). As we have found throughout our experiments, the 10^{-6} inoculum elicits a significantly smaller response in antigen and antibody production than more concentrated inocula, and in accordance with this the anticomplementary property develops to a less degree in the lungs of this group.

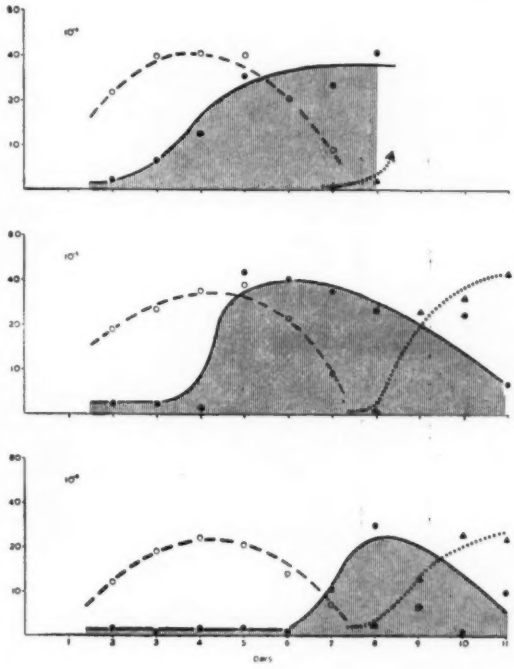

Fig. 1. Development of anticomplementary property of mouse lung after infection with MEL (A) virus in dilutions of 10^{-4}, 10^{-5} and 10^{-6}.

Antigen titre ----------; percentage anticomplementary property ——————; antibody titre ·····. Shaded areas represent regions of antigen-antibody complex formation.

These findings indicate that an antigen to be used as a standard in CF-tests should be prepared from mouse lungs harvested on the second, or at the latest, on the third day after infection, since after this time the lungs contain an antigen-antibody complex as well as free antigen.

Absence of correlation between CF-antigen yield and degree of lung consolidation.

The assumption that the consolidated areas of influenza-infected mouse lungs contain most of the CF-antigen and should be used preferentially in the preparation of standard antigens (Hoyle, 1948), was tested in two ways.

Firstly, the consolidated areas of a group of ten lungs were dissected from the non-consolidated areas, and each pool was ground up and suspended in Ca-Mg-saline to make approximately equal concentrations of lung tissue. After standard absorption with human red blood cells. the CF-antigen titres of the two pools were found to be identical. Although only a very crude test, it did indicate that CF-antigen production was not localized in areas of lung consolidation.

Secondly, a large number of 6–7 weeks old mice received intranasal inoculations of living MEL (A) virus in dilutions of 10^0 to 10^{-5}. For five days after infection, five mice from each group were killed daily. Lung lesions were recorded and the CF-antigen titre of each mouse lung suspension estimated.

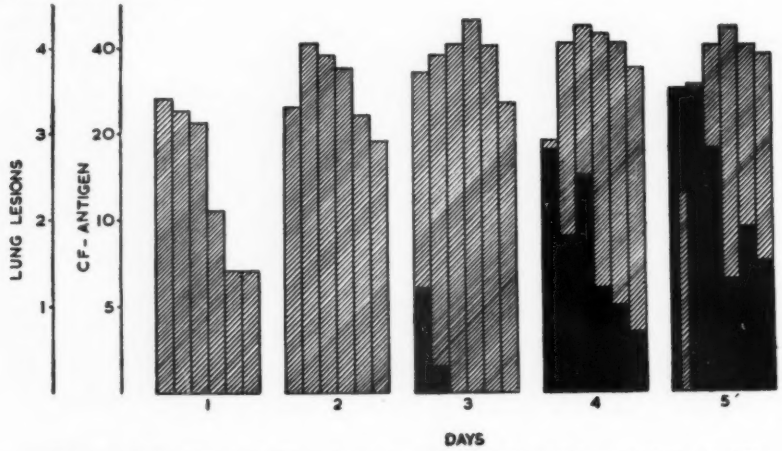

Fig. 2. Comparison of CF-antigen titre (hatched) and lung lesions (solid) after infection with MEL (A) virus in dilutions of 10^0 to 10^{-5}.

In Fig. 2, the mean of daily CF-antigen titres and the average lung consolidation of each group are plotted for the 5 days. There is a definite time lag between CF-antigen production and the appearance of lesions. Although antigen is detectable in all groups on the first day, and is at a high level for the following four days, lung lesions begin to appear only on the third day (and then only with concentrated inocula), and are still increasing on the 5th day. This more than suggests an independent development of the two properties, and indicates that it is not necessary to aim at the production of lung lesions in the preparation of a CF-antigen.

Optimal production of CF-antigen.

An examination of Fig. 2 shows a good yield of CF-antigen as early as the second day for all inocula, and a maximal level is maintained until day 5. The one exception is the 10^0 group which has reached its peak on the first day and then declines, without approaching the maxima attained by other groups. This phenomenon has already been discussed (Fazekas de St.Groth and Donnelley, 1950a), but must be stressed. In other words, a highly infective inoculum should not be used for the production in mice of CF-antigen. From the range of possibilities the following procedure was adopted in the preparation of a standard CF-antigen.

Mice were inoculated with a 1:100 dilution of fresh high titre allantoic fluid and the lungs harvested on the second day, before the appearance of visible lesions. This early harvesting ensured that the preparation would not be anticomplementary. The CF-antigen titre of such pools of mouse lung ranged between 30 and 40. A LEE (B) CF-antigen was prepared in the same way, but titres were somewhat lower (15–20).

Antiserum.

Optimal production of CF-antibody.

Initially the ferret was used as the source of CF-antibody, because it provided large amounts of high titre serum. The intranasal inoculation of 0·5 ml. of MEL (A) and LEE (B) viruses resulted, on the average, in the appearance of specific antibody by the 8th day, and maximum titres (30–80 for MEL (A) and 20–40 for LEE (B)) by the 10th or 11th day. Accordingly our ferrets were bled out from the subclavian vessels on the 11th day.

However, due to the non-specific reactions shown by nearly all ferret sera (see below) it was decided to use mouse serum, whose high specificity more than compensated for the tedious collecting and pooling of small volumes of blood.

Results of experiments on the production of CF-antibody in mice have been presented in paper III of the series (Fazekas de St.Groth and Donnelley, 1950b). The dilutions of MEL (A) virus used were 10^{-4} to 10^{-9}, but the high death rate (sometimes 100 p.c. for a 10^{-4} inoculum and 80 p.c. for a 10^{-5} inoculum) made these dilutions quite impracticable. The 10^{-6} dilution, (approximately 0·3 mouse ID_{50}[2]) although giving significantly lower antibody titres was selected because of the good survival of the animals (90–94 p.c.). The mice were bled on the 12th day after infection, at which time the CF-antibody had reached its maximal level (15–30). LEE (B) antiserum was prepared similarly by infecting mice with a 10^{-4} dilution of fresh LEE allantoic fluid (approximately 0·5 mouse ID_{50}) and collecting blood on the 12th day. With this inoculum 90 p.c. of the animals survived, and the pooled sera gave titres between 10 and 20.

Treatment of antisera.

The serum taken from the contracted clots was inactivated at 56° C. for 30 minutes and merthiolate (1:10,000) added. Serum to be kept for long periods was stored at —10° C., but ordinarily it was stored at 4° C.

The effect of heat upon the non-specific and specific CF-reactions was investigated. Samples of serum were diluted 1:5 in Ca-Mg-saline, and heated in the water bath at 56° C. for 20 minutes, 60° C. for 20 minutes, 62° C. for 15 minutes and 65° C. for 15 minutes. The CF-reaction against the homologous and heterologous antigen was then tested.

Ferret sera were variable in their non-specific reactions, most of them reacting markedly with the heterologous antigens (titres 15-40), although not being anticomplementary. This non-specific reactivity was not reduced even by heating at 65° C., when 50–75 p.c. of the specific antibody was destroyed. The small number of ferret sera with a low (5–10) or absent (<5) false positive reaction were the only ones used as standards in our tests.

Mouse sera on the other hand showed very clear-cut reactions. With the exception of old sera, they did not react at all (<2) with the heterologous antigen even after heating only at 56° C. for 20 minutes. When present, the non-specific reaction was completely eliminated by heating at 60° C. for 20 minutes or the equivalent, 62° C. for 15 minutes. No apparent reduction in the specific CF-antibody titre occurred at these temperatures (c.f. the anti-haemagglutinin which was reduced by $0·4 \pm 0·1$ $\log_2$ units (Fazekas de St.Groth and Donnelley, 1950b). A 75 p.c. reduction in CF-antibody was caused by heating at 65° C. for 15 minutes. This is in

[2] ID_{50} (infective dose) is the amount of virus which causes an average 50 p.c. lung consolidation on the 7th day after intranasal instillation.

agreement with the results of Casals and Palacios (1941) who, working with encephalitic viruses, found that mouse sera responded in much the same way to heat treatment; however, Fulton and Dumbell (1949) noted a rapid destruction of antibodies against the virus particle at 60° C.

As a large number of mouse serum samples had to be tested for both anti-haemagglutinin and CF-antibody, all samples were heated at 62° C. for 15 minutes, which inactivated the non-specific inhibitor interfering with anti-haemagglutinin titrations, and did not affect the CF-antibody titre.

Complement.

Complement was always titrated on the day of the test. Dilutions from 1:50 to 1:150 were made in Ca-Mg-saline. Unit volumes of these dilutions were incubated with two volumes of Ca-Mg-saline in the 37° C. waterbath for 60 minutes. One volume of sensitized sheep cells was then added to each tube, and the tubes incubated at 37° C. for a further 30 minutes. The dilution of complement giving 50 p.c. haemolysis (1 HD_{50}) was taken as the end-point, and 3 HD_{50} were used in the test.

At first fresh complement was always used for our tests but later it was found that complement stored at 4° C. maintained its titre for at least 3 days. No preservative was used.

As was mentioned above in the section on the CF-antigen, the effect of different dilutions of antigens on the titre of complement was always determined in the complement titration, and the antigens were used in a starting dilution which was not anticomplementary. Sera were tested in the same way. So, by "diluting out" the anticomplementary property of antigens and sera, a minimal excess of complement could be used, thus making the test highly sensitive.

Haemolytic System.

A mixture of freshly washed sheep cells and rabbit anti-sheep haemolysin was made up in Ca-Mg-saline, so that the final concentration of cells was 3 p.c., and of haemolysin 8 HD_{50}. The mixture was incubated at 37° C. for 30 minutes and kept at 4° C. when not in use. Sensitized cells could be used satisfactorily for 2 days.

Standardization of Antigen and Antiserum.

Chessboard titrations of CF-antigen and antiserum showed maximal titres for antigen and antibody against the lowest dilution of antiserum and antigen (1:5 in both cases). The prozone phenomenon (Hoyle and Fairbrother, 1937b; Lush and Burnet, 1937; Nigg, Crowley and Wilson, 1941) was not encountered with these dilutions. The above finding applied to ferret and mouse serum and also to human serum. Hence the optimal dilution for standard antigens and sera was 1:5.

PERFORMANCE OF TEST.

Small stainless-steel racks, 21 × 8 cm., were made to hold, in a strictly vertical position, 48 glass tubes, 7·5 mm. long and of 8 mm. internal diameter. A great advantage of such compact racks was the convenience of handling, especially in the delivery of drops to individual tubes.

An ordinary Pasteur pipette delivering 25 drops per ml. of Ca-Mg-saline was used throughout the tests. The fact that size of drop varies with surface tension was not considered a complication as the drop volume was consistent for each reagent. Since the variation in the size of drops is minimal, a high degree of uniformity can be achieved by this technique (see section on "Accuracy"). Doubling dilutions of the test samples (antigen or serum) were made in 2 drop volumes in Ca-Mg-saline. To these were added 2 drops of comple-

ment diluted to contain 3 HD_{50}, and 2 drops of standardized antibody or antigen, in that order. After thorough shaking, the racks were incubated in the waterbath for 60 minutes at 37° C., with a further shaking at half-time. Two drops of sensitized cells were added to each tube, and the racks shaken and returned to the waterbath for 30 minutes. Shaking at least once during the lysis period to disperse any unlysed cells was found to be essential. On removal from the waterbath the titrations were read. Values of 4, 3, 2, 1 and 0, representing 0, 25, 50, 75 and 100 p.c. lysis were read by eye, in terms of turbidity. With practice a comparison with standards was found unnecessary; no finer gradations were attempted. Such readings could be carried out quickly and with reproducible results (see below). The 50 p.c. or 2 value, being much more accurate than the 100 p.c. (Brooks, 1920), was taken as the end-point, and titres were expressed in terms of the initial dilution of test material in unit volume, not in the total volume of the test.

Micro-technique.

In cases where extremely small amounts of test material were available, e.g. bronchial washings or serum from tail-bleedings of mice, a technique using a single drop of antigen, antiserum, complement and sensitized cells was tried. Doubling dilutions of the test samples were made in Ca-Mg-saline and complement, so that mixing and transferring could be done in 2 drop rather than 1 drop volumes. This single drop method proved to be so convenient and at the same time so accurate, that it was adopted later for all CF-titrations.

Accuracy of technique.

In experiments of our main study, samples of antigen and serum were collected daily. It was convenient to store these for several days and titrate a large number at once. In order to see whether the samples would keep without deterioration and whether reliable results could be obtained, the reproducibility of the method was assessed experimentally.

Fifty samples of mouse lung were titrated for CF-antigen on one day and again 3 days later. The tests were done by the 2 drop technique, and the standard deviation of replicate results was found to be $\pm 0{\cdot}33$ $\log_2$ units.

To assess the accuracy of the method, a preparation of CF-antigen of unknown titre was made into different dilutions by a colleague, and 30 samples referred for titration. A statistical analysis of the results gave a standard deviation $\left(\sigma = \sqrt{\frac{\Sigma \bar{x}^2}{n-1}}\right)$ of $\pm 0{\cdot}285$ $\log_2$ units.

DISCUSSION.

Several points of difference from other methods (Fairbrother and Hoyle, 1937; Francis, Magill, Rickard and Beck, 1937; Lennette and Horsfall, 1940; Eaton and Rickard, 1941; Hoyle, 1945; Rice, 1947; Hoyle, 1948) have been included in the preparation of an influenzal CF-antigen and in the complement-fixation technique described in the present paper.

All workers have recognized that the CF-antigen and the virus particle are separable by a variety of treatments, in particular, high speed centrifugation, but only a few (Henle *et al.*, 1944; Sigel, Allen, Williams and Girardi,

1949) have made a strict practice of removing the virus completely from CF-antigen preparations for serological or epidemiological purposes. This seems surprising in view of the fact that in titrations of serum, strain specific phenomena (Hoyle, 1945; Wiener *et al.*, 1946; Hoyle and Fairbrother, 1947) and possibly false positive reactions in immune or vaccinated populations, may be accounted for by the presence of virus in CF-antigen preparations. In this study, red blood cell absorption was always employed because it was simpler and more practicable when working with large numbers of samples of small volume. Further, an infectivity test for residual virus does not seem as satisfactory as a haemagglutination test. After treatments, especially chemical ones, designed to purify antigens, virus may be present in a non-infective form but with its haemagglutinating capacity still intact, and it is the latter which is related to the viral CF-reaction. For this reason, CF-antigens were always checked to make certain that their haemagglutinin titre was less than 2.

Also, in the selection of optimal conditions for the preparation of a CF-antigen from mouse lung, it was found that consistently lower titres resulted from undiluted inocula (100,000 mouse ID_{50}) than from smaller inocula (10,000 to 1 mouse ID_{50}). The phenomenon was discussed in paper II of the series (Fazekas de St.Groth and Donnelley, 1950a). Dilutions of inocula of 10^{-1} to 10^{-5} gave comparable titres, and a 10^{-2} dilution was chosen, the infected lungs being harvested after 2 days.

Conclusive evidence for the absence of correlation between CF-antigen yield and degree of lung consolidation showed that aiming at the production of gross lesions (about the fourth day) was not necessary, and was in fact undesirable, in view of the marked anticomplementary property developing in the lungs by this time.

The anticomplementary property of infected mouse lung seemed to indicate an *in vivo* combination of antigen and antibody, and presumably both antigens (virus and CF-antigen) and their antibodies would be concerned in the reaction. If antigen is capable of "fixing" antibody in this way, then the time discrepancy in the detection of antibody in the serum of infected or immunized mice can be explained. In Paper III (Fazekas de St.Groth and Donnelley, 1950b) we followed the development of antibodies in mice. In infected mice anti-haemagglutinin appeared in the serum only after 7 or 8 days, whereas in vaccinated mice, i.e. mice in which no multiplication of virus occurred, anti-haemagglutinin was detected as early as the third or fourth day. This points to a neutralization of developing antibody by the multiplying virus in infected mice, with a consequent delay of 4 days in its appearance in the circulation.

The advantages of the ferret as a large, easily infectible experimental animal were outweighed, in our experience, by the non-specificity of most antisera. Heating to 65° C. did not affect the non-specific reaction, even though the specific antibody titre was reduced by 75 p.c. Other attempts to eliminate

these non-specific reactions (Lennette and Horsfall, 1941) were unsuccessful. The tedious collecting of mouse sera proved well worthwhile as they gave such clear-cut reactions. Rarely were non-specific reactions found, and when present were eliminated by heating at 60° C. for 20 minutes or 62° C. for 15 minutes, at which temperatures reduction in specific CF-antibody was not apparent. Although antibody titres were relatively low, mouse sera could be used satisfactorily in a 1 : 5 dilution.

The standard amount of complement was 3 HD_{50}, which would be equivalent to 1·3 minimal haemolytic doses. Use of such a small excess was made possible because the anticomplementary property of antigens and sera was diluted out, the lowest dilution used in the tests in each case being 1 : 5. Thus a high sensitivity could be obtained.

Little need be said about the actual performance of the test. The technique was modified to cope easily with large numbers of titrations, and with test materials of small volume. It is felt that this aim was satisfactorily realized as several hundred tests could be carried out in one day. At the same time a high degree of reproducibility and accuracy was maintained, the standard deviation of replicate results on different days of testing being $\pm 0{\cdot}33$ $\log_2$ units, and the titration of a large number of unknown dilutions of one antigen performed on one day with the same reagents gave a standard deviation of $\pm 0{\cdot}285$ $\log_2$ units. Thus any two readings obtained by the improved CF-test which are 0·66 $\log_2$ units apart are significantly different at the 5 p.c. level, and those 1·0 $\log_2$ units (one tube) apart are different with a probability of 99/100.

Later work has proved this modified technique to be eminently suitable for the titration of human sera.

SUMMARY.

The small complement-fixing antigen (CF-antigen) of influenza was optimally prepared from mouse lung 2 days after infection with 10^6 egg ID_{50} of either A or B type virus. By this stage, neither visible consolidation nor anticomplementary property had developed in the lungs.

The virus particle interfering in the complement-fixation test was removed by absorption with human red blood cells.

Because of marked non-specific reactions which could not be eliminated by heat-inactivation ferret sera proved unsuitable as standards. Sera of experimentally infected mice, heated at 62° C. for 15 minutes, were completely devoid of non-specific reactions. This treatment did not reduce specific antibody titres.

Technical details are presented of an improved, small volume complement-fixation test for the titration of either antigen or antibody. The method is accurate within $\pm 0{\cdot}285$ $\log_2$ units and reproducible within $\pm 0{\cdot}33$ $\log_2$ units.

Acknowledgment. I am indebted to Dr. S. Fazekas de St.Groth for his advice and criticism.

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THE INOCULATION, CULTIVATION AND ENUMERATION OF BACTERIA ON GLASS SURFACES.

METHODS FOR DISINFECTION STUDIES

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(Accepted for publication 22nd December, 1950.)

Experiments are at present being conducted in this laboratory on the effect of atmospheric humidity on the survival of bacteria on glass surfaces.

A great deal of the past work on these and similar studies has involved indirect methods of culture, i.e., the organisms have been removed from the surfaces by some means and transferred to suitable culture media to test for viability.

The need was felt for a simpler and more direct method of estimating the viable organisms on such surfaces. In preliminary experiments it was observed that bacteria, deposited and allowed to dry on smooth glass surfaces, often, though not always, tended to spread and form confluent growth at the glass-agar interfaces when these surfaces were embedded in a nutrient agar medium; from confluent growth it is impossible to obtain a valid viable count of the organisms. With suitably ground glass surfaces, however, the organisms invariably developed as discrete colonies, thus permitting valid viable counts to be made. It was also shown that by means of a bacterial mist, the organisms could be deposited on ground glass surfaces so that their distribution was or approached that of a Poisson pattern. Disinfection by atmospheric humidity of ground glass surfaces inoculated by such a procedure is being studied.

It is at present uncertain how much work is required to obtain reliable data in this disinfection study. For example, it has been found that while recoveries of bacteria on glass surfaces which are cultured immediately after inoculation are not affected by any one of a number of methods used to clean the glass, the recoveries after exposure, particularly to relatively humid atmospheres, are markedly modified by the cleaning methods and also probably by the initial composition of the glass. It is evident that these factors need investigation before humidity effects, *per se*, can be assessed. There are, however, certain features of the methods (referred to in the title of this paper) which are considered to be sound and worth recording at this juncture as they appear to have application in a number of related fields.

The test organism for most of the work being done at present is a viridans streptococcus.

Methods.

Glass slides.

Ordinary microscope slides (3" × 1") are ground on one surface with carborundum powder "150".

Before use the slides are cleaned in nitric acid, rinsed in tap and distilled water and finally dried in an oven for a few minutes.

Inoculation of the slides.

In recent years much work has been done on bacterial mists and the possibility was entertained of using such a means for inoculating the glass surfaces.

Preliminary experiments showed that when broth suspensions of bacteria were atomized into a chamber, the organisms impinged on the surfaces of agar plates and glass slides when these surfaces were exposed towards the floor of the chamber. Completely negative recoveries were obtained, however, when the bacteria were suspended in 1·0 p.c. sodium chloride. With this suspending fluid, recoveries could be obtained by exposing the surfaces so that the bacteria were free to fall on them. The difference in recoveries between two surfaces exposed at the same level to a saline mist in the chamber, but facing in the one instance towards the floor and in the other towards the ceiling, was most striking. No such difference was observed with organisms suspended in beef heart broth or in peptone solutions. In the work done so far, these latter fluids have been used for suspending the bacteria and the following is an outline of the procedure adopted for inoculating the slides.

The slides are arranged radially on the circumference of a disc which is fitted into the ceiling of a chamber so that the test surfaces of the slides are exposed towards the floor of the chamber. The bacterial mist from an atomized broth suspension of the organism is introduced into the chamber and the disc is slowly rotated (about one rev./second). The distribution of the bacteria on the glass surfaces attained by this method of inoculation is discussed in a later section of this paper.

Cultivation of the organisms.

Petri dishes are poured with a layer of agar which is allowed to set. Immediately before culturing, a thinner layer of agar at 45°C. is poured over the surface of the initial layer. The inoculated slides are placed with their ground surfaces downwards in the liquid agar, and the dishes are incubated after this agar has set. The method is suitable for organisms such as the viridans streptococcus, but for more aerobic organisms it is necessary to embed the slides so that the inoculated surfaces face the free surface of the agar. In either case the organisms develop as discrete colonies at the glass-agar interfaces.

Enumeration of the colonies.

An alternative method to that of counting the colonies *in situ* was developed, for simple though this is with small numbers, it is inconvenient and tedious when the numbers are large.

It was found that the slides could be removed easily from the agar, and that when this was done carefully, most of the mass of each bacterial colony retained its position on the slide. Thus, freed from the agar, the colonies could be stained and counted more easily. Such preparations can however be photographed by a simple technique and the following routine is adopted for most of the counting.

The slides are removed from the agar, placed in absolute alcohol for a few seconds, then dipped in a weak collodion solution and allowed to dry. The thin collodion film prevents the colonies from becoming detached from the slides in the subsequent staining procedure. The slides are placed in ammoniacal silver carbonate solution for about 30 minutes, rinsed in distilled water, dipped in dilute formalin, washed in tap water and allowed to dry. The resulting black colonies on a translucent background are photographed by direct contact printing (or enlargements may be made), thus obtaining a sharp negative image of the preparation and at the same time a convenient and permanent record of the counts. Fig. 1 shows two examples of such preparations. In practice, the edges of the slides are masked so that only the colonies within a well defined border are counted.

The sharp images of the colonies facilitate their accurate enumeration and the counting error associated with the method is small. In several tests of this error the widest divergence recorded between replicate prints expressed as a percentage of the mean, was approximately 3 p.c. Counts of as high as approximately 800 colonies per slide can be made with this accuracy. Above this figure counting becomes increasingly difficult and inaccurate.

Fig. 1. Negative images of slide preparations of colonies of a viridans streptococcus (see text).

EXPERIMENTAL.

Adherence of organisms and colonies to the slides.

Factors which are of fundamental importance to the method are those involving adherence of the organisms and colonies to the slides in the interval between the inoculation of the slides and enumeration of the colonies. Possible sources of error and the obesrvations made on them include the following:

Loss of organisms from slides immediately after inoculation. It was shown that such treatments as rapping the slides on the bench or waving them vigorously in the air, resulted in no measurable loss of organisms.

Loss of organisms from slides during culture. Occasionally, organisms become detached from the slide surfaces as the slides are placed in the liquid agar and these organisms colonise in the free agar in the vicinity of the slides. The numbers of organisms which are lost from the slides in this manner are, however, insignificant.

Loss of colonies during removal of slides from the agar. The colonies appear to have a greater affinity for the glass than for the agar and it is easily observed that most of the mass of each bacterial colony adheres to the slide as it is separated from the agar.

Loss of colonies during the staining procedure. This can be a source of serious error, but is prevented by means of a collodion film as described earlier in this paper.

Summarizing these observations, it may be stated that there is no evidence for significant losses of organisms or colonies in the interval between the inoculation of the slides and the enumeration of the colonies.

The possibility of organisms becoming detached from the slides during disinfection processes can be investigated only in relation to the particular problem being studied and a consideration of it is beyond the object of this paper.

Distribution of the organisms on the slides.

On theoretical grounds, it was considered likely that the variance of colony counts of replicate slides inoculated by means of the bacterial mist would be that of a Poisson distribution.

In a group of early experiments it was found that 80 p.c. of 120 sets of parallel counts gave χ^2 values the corresponding probability (P) values of which were between 0·01 and 0·99 while the remaining 20 p.c. showed some degree of excess variation (i.e. $P < 0{\cdot}01$). While these results show significant departure from Poisson distribution, they are considered to reveal a degree of randomisation not unsatisfactory for quantitative studies.

More recent experiments appear to be yielding a lower incidence of excessive variation, indicating perhaps, a general improvement in technique.

DISCUSSION.

The methods most nearly approaching those described in this paper that could be found in the literature were those employed by Buchbinder and Phelps (1941) in their studies on the survival of bacteria on filter paper. These workers inoculated filter paper by exposing it on the floor of a chamber to a falling bacterial mist, and cultured the filter paper in much the same way as that described for the slides in this paper. They found that unequal distribution of the bacteria on the filter papers was the chief source of error in their studies. Such was the experience with the ground glass slides in this laboratory until the rotating disc was introduced into the method. Rotation of the slides tends to obviate the effects of fluctuations and gradients in the concentration of the mist in the chamber.

As yet, no investigation has been made of the factors responsible for the adherence of the organisms to surfaces so placed that they can be inoculated only by organisms impinging on them from below.

Methods which ensure a discrete growth of colonies at the interface of *smooth* glass and agar are being investigated, but as yet have not been satisfactorily developed.

The possibility of the application of the methods described here to studies of other forms of vapour-phase disinfection and of disinfection by radiant energy is thought to be worth consideration.

The inoculation procedure described was chosen for a number of reasons which are of no direct concern in this present paper. It is conceivable that other methods such as the spreading of measured drops of bacterial suspension over the surfaces may be useful in certain studies.

Glass slides and coverslips are commonly employed in investigations such as the one for which the methods described here were designed. In the introduction to this paper are mentioned some observations which illustrate the danger of regarding these surfaces as "inert" in such disinfection processes. It is felt that other materials such as pure quartz or perhaps some of the plastics could be substituted for glass in the methods described and that these materials may be more suitable for certain disinfection studies.

SUMMARY.

Methods have been described for the inoculation, cultivation and enumeration of bacteria on glass surfaces.

These methods are at present being employed in measuring the effect of atmospheric humidity on the survival of bacteria.

Attention is directed to the possible application of the methods in related fields.

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THE POSSIBILITY OF THE DUAL NATURE OF SYMPATHETIC GANGLION CELLS III.

by F. H. SHAW[1], MONICA MacCALLUM, D. J. DEWHURST, AND J. F. MAINLAND

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(Accepted for publication 15th December, 1950.)

In two earlier papers (Shaw, 1948, 1949) it was suggested that there were two pharmacologically distinct groups of cells in sympathetic ganglia, one of which innervated those fibres which constricted vessels (called the C cells) and one which innervated fibres which, liberating adrenaline at their endings, nevertheless dilated vessels (the D cells). The evidence for such a suggestion may be summarized as follows. Nicotine, by stimulating the sympathetic ganglia, produces a rise in blood pressure. Acetylcholine in an atropinized animal gives similar results. But when this animal is also yohimbinized, nicotine does not produce a fall in blood pressure (the so-called vasomotor reversal) whilst acetylcholine does. Therefore, there is a difference between the action of acetylcholine and nicotine on the ganglion cells. The plan of the past and present experiments should be evident from Fig. 1.

Perhaps the crucial experimental point is whether in an atropinized, yohimbinized animal, the fall of blood pressure due to an injection of acetylcholine is the result of post-ganglionic liberation of adrenaline, or of the acetylcholine "breaking through" the atropine (muscarinic action). This aspect has already been dealt with in a previous paper (Shaw, 1949). It is considered again in this paper (see action of ergotamine). As has been mentioned, the unmasking of the dilator action of the sympathetic has been brought about by peripheral antagonism,

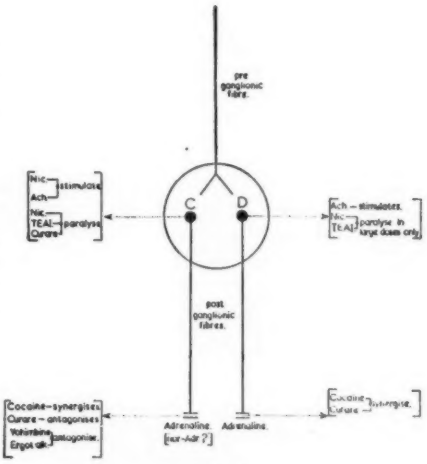

Fig. 1.

[1] This work was assisted by a grant from the National Health and Medical Research Council.

using yohimbine. It is also possible to bring about this unmasking by central (ganglionic) action. If the animal has received large doses of nicotine, so that the ganglionic cells are no longer stimulated by nicotine (i.e. injection of nicotine no longer produces a rise in blood pressure), then the injection of acetylcholine (after atropine) will give a pure fall in blood pressure. Thus it is said that at this stage the C cells only are paralysed by nicotine; further administration of nicotine will paralyse the D cells also. Curare behaves in a similar manner to nicotine. In this paper the action of other ganglion paralysants is investigated further.

Finally, the whole of the evidence so far presented in favour of the theory is pharmacological in nature, and this has its limitations. Accordingly in the first section of this paper confirmatory evidence of a like nature is presented, whilst in the second section an attempt has been made to present evidence of a more substantial nature by investigating electrically the response of the ganglion to stimulation (and paralysis) by the series of drugs used in connection with this work. The results may be summarized at present by saying that, whilst there is no evidence that there are C and D cells in the ganglia, we have shown electrically that acetylcholine does stimulate cells in the ganglia which nicotine does not and that all cells are not equally paralysed by pharmacodynamic agents, whether the test stimulation be natural (stimulation of preganglionic fibres), or artificial (by injected acetylcholine etc.).

EXPERIMENTAL.

METHODS.

Except where otherwise stated all the results in this paper were obtained by means of the methods listed in an earlier paper (Shaw, 1948).

For the electrical experiments the set up was as follows: In the cat the pre- and postganglionic fibres of the superior cervical ganglion were carefully dissected. Platinum electrodes were placed on the preganglionic fibres and stimulation provided by a square wave generator, enabling the pulse amplitude, duration, and repetition rate to be varied at will. Usually two repetition rates were chosen of 4 per second and 10 per second, (1 and 2 in protocol). Platinum pick-up electrodes were placed on the postganglionic fibres and the impulses were reproduced, after suitable amplification by an Offner type 140 amplifier, on a Cossor 339 oscillograph, loudspeaker and recording A.C. voltmeter. In general it was found most convenient to record synchronous discharges (from electrical stimulation) by means of the CRO, and asynchronous and repetitive discharges (from drug injection) by means of the recording A.C. voltmeter. Drugs were injected via the saphenous vein. Artificial respiration was only used when necessary (e.g. with curare). The incision was filled with paraffin oil to prevent the nerves from drying off.

PHARMACOLOGICAL EXPERIMENTS.

Tetraethylammonium iodide (TEA I) and tetramethyl ammonium iodide (TMA I). In view of the very extensive knowledge we now have of the pharmacology of TEA I, due to the work of Acheson and Moe (1946), it was thought desirable to investigate the ganglionic blocking powers of this compound with respect to the above theory.

TEA I (dose 15-20 mg./kg.) blocks the "nicotine" rise of blood pressure due to acetylcholine (in an atropinized animal) with the result that acetylcholine produces only a fall. This

fall was increased by cocaine curare and ergotamine tartrate. This agrees perfectly with the theory, in that these results parallel those obtained with nicotine i.e. TEA I paralyses the C cells but leaves the D cells unaffected. Hence, after TEA I, acetylcholine stimulates the D cells only; this results in a release of "dilator" adrenaline, confirmed by the fact that the subsequent fall in blood pressure is potentiated by cocaine, curare and ergotamine. (Fig. 1).

Tetramethylammonium iodide gave much less definite results. As has been stated before, the nicotinic action of acetylcholine is only seldom a pure rise in blood pressure. There is usually an initial fall and sometimes a terminal fall. TMA I most often decreased the rise and increased the fall. Only rarely was the pressor element abolished completely. This means that the compound has only slight paralysing action on the C cells.

Some other interesting properties of TEA I may be mentioned, as they appear to be given by all quaternary ammonium compounds: e.g. curare, quinine dimethiodide, (Shaw, 1949). The fall in blood pressure induced by TEA I is reduced and finally abolished by repeated doses. This fall in blood pressure is also lessened or abolished by cocaine. The pressor action of nicotine is abolished.

The fall in blood pressure due to TEA I is uninfluenced by benadryl and pyribenzamine, so histamine does not play a part. Large doses of TEA I produce a rise in blood pressure which is reduced, but not abolished, by yohimbine, and curare. We may say that part of this rise is due to stimulation of the adrenals or of the C cells in the sympathetic ganglia which, however, must be counted very insensitive.

TMA I (dose = 0·25 — 0·5 mg./kg.) itself raises the blood pressure, presumably by stimulating the C cells, as this rise is abolished by TEA I or curare. A fall after these two drugs has not been obtained, so presumably TMA I cannot stimulate the D cells. In this action on the ganglion TMA I resembles nicotine in so far as it is also able to paralyse the C cells and prevent a rise on injection of nicotine. Another resemblance of TMA I to nicotine is that TMA I rise is abolished (even reversed) after yohimbine.

As is well known the pressor response of adrenaline is changed to a depressor when the animal is yohimbinized (0·3 mg./kg.)—the so called "vasomotor reversal". We have found that this fall in blood pressure is changed to a rise when either TEA I or TMA I is administered. This now appears to be a common property of all quaternary ammonium compounds (Shaw, 1949).

Ergotamine tartrate (dose 0·15 — 0·4 mg./kg.) and ephedrine HCl (dose 0·1 — 0·3 mg./kg.). It was pointed out in the introduction that the fall in blood pressure following an injection of acetylcholine into an atropinized and nicotinized cat was due to liberation of adrenaline from dilator fibres and not due to the acetylcholine "breaking through" the atropine. Confirmation of this hypothesis was given by the finding that the fall was potentiated by cocaine. In view of this action it would be interesting to investigate the action of ergotamine and ephedrine on this fall.

In a yohimbinized cat, in which the vasomotor reversal response to adrenaline is evident, ergotamine and ephedrine diminish or abolish the fall due to adrenaline, and may often restore the rise in blood pressure (see also Koppanyi, 1939). It is difficult to find an explanation for this, especially in the case of ergotamine. Why should an agent which itself brings about the vasomotor reversal, antagonize another compound (yohimbine) which has a similar power? The difficulty is not eased by the fact that contrary to the findings of Koppanyi (1939) ergotamine increases the fall in blood pressure due to acetylcholine in an atropinized animal previously treated with curare or TEA I (Fig. 2). This latter result of course supports the idea that the fall in blood pressure is due to liberation of adrenaline from postganglionic nerve endings. This recalls the results of Koppanyi (1939) and Linegar (1940) where adrenaline vasomotor reversals brought about by ergotamine were nullified or reversed by large doses (10 mg.) of atropine. In all our experiments with acetylcholine the animals were deeply atropinized. Such antagonism has also been shown by Stehle and Melville (1949).

Eserine (dose 0·25 — 1 mg./kg.). In terms of the theory postulated in the introduction it is difficult to foretell the effects of eserine. It may increase the stimulating effects of acetylcholine on the D cells of the ganglia; it may lessen the action of the atropine and so assist in the breaking through of the acetylcholine (muscarinic action). The actual results are that the depressor action of acetylcholine after nicotine, TEA I and curare is abolished and turned to a rise. However, if the acetylcholine dose after the eserine is reduced to one tenth, then a fall is still the result (Fig. 3). The interpretation for this is that the eserine antagonizes the TEA I and curare at the ganglia, and also permits a greater amount of acetylcholine (due to non-destruction in the blood) to reach the ganglia, here, perhaps by competition, acetylcholine is able to displace the TEA I and curare, and so stimulate the C cells. When the dose is reduced to one tenth, only the D cells are stimulated; as we have shown in an earlier paper, that these cells are more sensitive (Shaw, 1948).

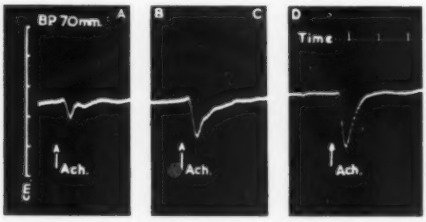

Fig. 2.

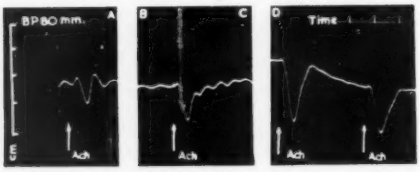

Fig. 3.

Fig. 2. Cat. Atropine 2·5 mg./kg. Ach. Acetylcholine 0·05 mg. Between A and B TEA I 4 mg./kg. Between C and D Cocaine 4 mg/kg. Time 30 seconds (in all Figures).

Fig. 3. Cat. Atropine 2 mg./kg. Ach. Acetylcholine 0·15 mg./kg. Between A and B TEA I 5 mg./kg. Between C and D ergotamine tartrate 0·2 mg./kg.

To summarize this section it may be said that there is a considerable amount of pharmacological evidence in favour of the theory postulated in the introduction. It is felt now that no good purpose would be served by piling up facts of a similar nature to bolster up the theory. It is felt that the attack must be made from an entirely new direction.

Electrical Activity.

The superior cervical ganglion of the cat was chosen as the test object. On stimulation of the preganglionic fibres there appears in the postganglionic fibres an action potential picture of three or four groups of spikes; that is to say that in all probability there are four sets of fibres of different diameters in the preganglionic group, which of course have different conduction velocities. The dispersion of the impulses depends on the distance of the stimulating electrodes from the ganglion. The impulses are relayed in the ganglion and pass down the postganglionic fibres, where little dispersion takes place as the distance between the ganglion and the pick-up electrodes is only of the order of a few millimetres.

It is unfortunate that little work has been done to map out the regions in the head supplied by the different sets of fibres. One must depend on the

work of Eccles (1935), Bishop (1932) and Rosenbleuth (1941). It may be said that the S_1 wave (Fig. 4) represents the nictitating membrane. It is proposed to map out the head region with respect to these waves at a later date. As it is impossible at present to isolate the fibres carrying impulses which will constrict or dilate vessels in the head region, it is not possible to confirm or refute exactly the postulated theory concerning the C or D cells of ganglia. However, if it could be shown that acetylcholine could initiate impulses in a ganglion sufficiently paralysed by nicotine, curare or TEA I whilst nicotine could not, then this would be the same as saying that there were at least two types of cells in the ganglia which could be distinguished pharmacologically. It would be very strong presumptive evidence for the presence of the D cells. This we have been able to do and the results are reported below. At the same time we have accumulated evidence which leads us to believe that the ganglionic cells behave very differently with each of the ganglionic paralysants, nicotine, curare, TEA I, antilusion (C_5) etc. This will form the subject matter of a separate paper.

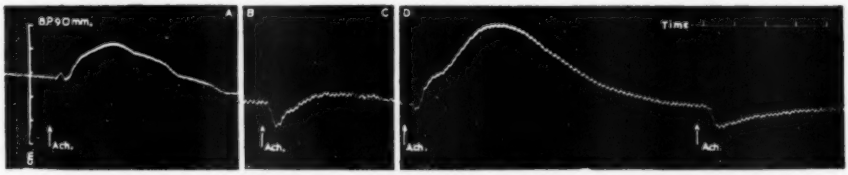

Fig. 4. Cat. Atropine 2 mg./kg. Ach. Acetylcholine 0·3 mg./kg., last dose of ach. 0·03 mg./kg. Between A and B, D tubocurarine (B.W.) 0·7 mg./kg. Between C and D eserine sulphate 0·7 mg./kg.

Results.

It has been found that 1-2 mg./kg. of nicotine injected into the saphenous vein stimulates the superior cervical ganglion causing a burst of impulses in the post-ganglionic fibres and contraction of the nictitating membrane (NM). In one cat out of ten no impulses were recorded nor did the nictitating membrane contract with doses as high as 3 mg./kg. If the nicotine dose is repeated at 2-3 minute intervals, after about the third dose the bursts are smaller and the NM does not contract so much. The paralysis increases over the next few doses. Again some cats are particularly easily paralysed by nicotine, as little as 2 mg. being sufficient.

A similar injection of acetylcholine (1-3 mg./kg.) produces a burst and a contraction of the NM. If the animal was unresponsive 0·5 mg. of eserine was administered, and a subsequent dose of acetylcholine provoked a burst and contraction. These dosages were insufficient to affect the NM either directly or *via* the adrenals. This was tested by administration after section of the postganglionic fibres.

In each experiment doses of nicotine and acetylcholine were adjusted as rapidly as possible until the burst due to nicotine was the same or slightly greater than that due to acetylcholine. The doses of acetylcholine and nicotine were then alternated at 2-3 minute intervals, and the contraction of the NM measured and the degree of bursts noted on an arbitrary scale (either by ear or on the recording voltmeter).

The results although definite have not the sharpness that one would desire. The most common cause of failure was the fact that the voluntary muscles of some cats were extremely

sensitive to nicotine with the result that the muscle potentials brought about by ear twitches etc. masked the presence of impulses in the postganglionic fibres. Remarkable insensitiveness to nicotine of those cells in the ganglion which innervated the set of fibres going to the NM was also noted. Finally and paradoxically, the NM would often contract without an accompanying burst of impulses in the post-ganglionics; this was attributed to the fact that the impulses were not picked up owing to the shunting by the inactive fibres. This has been proved by removing some of the fibres from the electrodes, and then bursts were obtained. The contraction was not due to direct action of nicotine on the NM, nor to adrenaline liberated from the adrenals, as it did not occur if the postganglionics were cut.

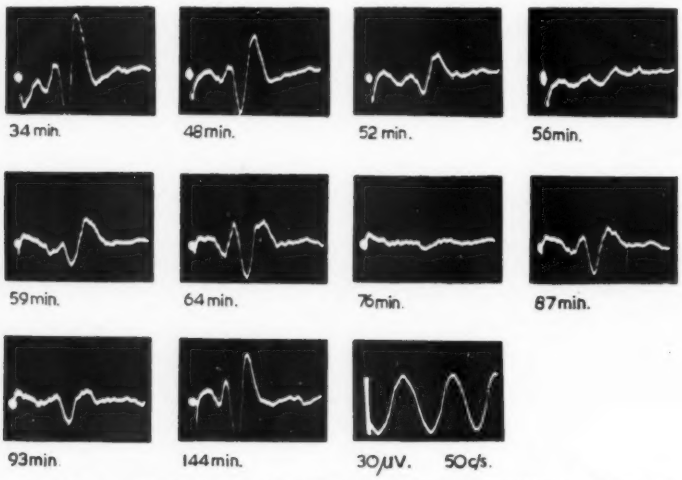

Fig. 5. Recordings on the CRO from the postganglionic fibres. To be read in conjunction with Fig. 6 and the protocol in Table 1.

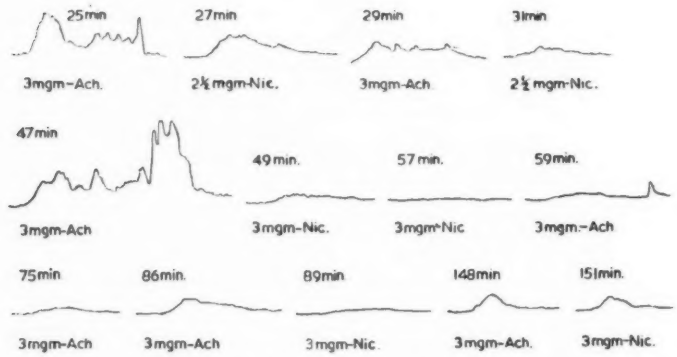

Fig. 6. Recordings on A.C. voltmeter of bursts from postganglionic fibres. To be read in conjunction with Fig. 5 and the protocol in Table 1.

TABLE 1.

Protocol of typical experiment.

Figures given below for the contraction of the nictitating membrane are on an arbitrary scale.

Time	Doses m.g.			Contraction of nictitating membrane				
				Elect stim.		Drug stim.	Burst	C.R.O. trace
Minutes	Nic.	Ach.	Es.	Frequency 1.	2.		Fig. 5	Fig. 4
12			½					
25		3				25	+++	
27	2½					33	++	
29		3				26	++	
31	2½					23	+	
33		3				14	+	
34	Elect. stim.							Good
35	2½					1		
37		3				5	+	
39			½					
47		3				42	+++	
48	Elect. stim.			15				Fair
49	3					27	+	
51		3				20	+	
52	Elect. stim.			11	16			Fair
53	3					1	0	
55		3				15	+	
56	Elect. stim.			2	6			Almost flat
57	3					0	0	
59		3				12	+	Fair

Between 60 minutes and 98 minutes acetylcholine and nicotine were given alternately every 3 minutes. This resulted in acetylcholine causing a burst of impulses but nicotine failed to cause any stimulation.

From 98 minutes to 143 minutes there was a period of rest for recovery.

143			½					
144	Elect. stim.							Good
148		3				24	++	
151	3					41	++	

Despite these difficulties about 14 experiments were carried out. Of these, in 9 cases we were able to show that acetylcholine still stimulated a nicotinized ganglion when nicotine itself had no effect. In 2 cases, whilst the initial control bursts due to nicotine were the same, the bursts due to acetylcholine were greater than those due to nicotine. In 2 cases the acetylcholine and nicotine sensitivity of the cells continued to be the same, and only in one case was the ganglion responding to nicotine but not to acetylcholine. These results show that in some animals a ganglion paralysed by nicotine is still irritable to acetylcholine, that is, presumably it contains D cells. Our electrical experiments were not as clear cut as the pharmacological ones, where a fall in blood pressure was always elicited by acetylcholine in a nicotinized animal. But it should be remembered that in the first case we were investigating one ganglion only, the superior cervical, whereas in the latter we have the whole sympathetic chain at our disposal, where it is more than likely that the population of D cells is relatively larger. Furthermore our negative results may be explained by assuming that the impulses from the D cells were carried in fibres separated from our electrodes by a large amount of inert shunting material. This finding was confirmed over and over again in the case of the NM.

The results of a typical experiment are shown in Figs. 5 and 6 and Table 1, which are to be read in conjunction.

Throughout this work records were kept of the threshold of the NM to acetylcholine, nicotine and electrical stimulation (of the preganglionics) during the course of paralysis by nicotine, curare, TEA I and antilusin. The results have been extraordinarily interesting but are as yet too incomplete to publish here. Suffice to say that the cells of the superior cervical ganglion supplying the post-ganglionic fibres which innervate the nictitating membrane, the orbit and the blood vessels (dilator or constrictor) do not react quantitatively in the same manner to all paralysants. In the future when one is testing an agent for paralytic activity on ganglionic transmission it will not be sufficient to employ only one test object, say the nictitating membrane, as is done at present. These results will be published in a later paper.

However, we have been able to show with the aforementioned paralysants that with increasing depth of paralysis there comes a time when nicotine is no longer able to generate a burst of impulses, whilst acetylcholine is still able to elicit response.

SUMMARY.

The following facts are discussed in connection with the theory that there are two distinct types of cells in sympathetic ganglia.

Tetraethylammonium iodide changes the "nicotine" rise of blood pressure due to acetylcholine to a fall, which is potentiated by cocaine.

The vaso-motor reversal of adrenaline is abolished by ergotamine tartrate and ephedrine HCl.

The action of eserine in an atropinized, yohimbinized animal is discussed.

A study of action potentials in the postganglionic fibres of the superior cervical ganglion, suggests that there are cells in the ganglion which are capable of stimulation by acetylcholine when "paralysed' by nicotine.

The action of tetraethylammonium iodide, nicotine, curare and antilusin (C_5) on the superior cervical ganglion has been studied electrically. The results suggest that there is present a group of cells which does not respond in the same manner, quantitatively, to these paralysants.

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